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Establishment of an Ex Situ Collection of *Glycyrrhiza glabra* L. as a Prerequisite for Field Cultivation in Bulgaria

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Abstract: *Glycyrrhiza glabra* L. (Fabaceae), commonly known as licorice, is a perennial medicinal plant. Its healing properties are due mainly to the secondary metabolites glycyrrhizin and flavonoids accumulated in the roots of plants aged 3 years or more. Overexploitation of licorice populations in Bulgaria led to their rapid decrease. The species is protected by the national Biodiversity Act. The present study aimed at establishing of an ex situ collection of *G. glabra* using plant material originating from its Bulgarian populations in order to evaluate the main characteristics of the cultivated plants and their potential use as a source of plant material for the creation of a plantation. Plants were obtained from stolon cuttings of donor wild-growing plants from three Bulgarian populations and then cultivated for 3 years in the experimental field plot. Plants originating from all three populations produced glycyrrhizin and flavonoids in similar concentrations under the controlled conditions of the ex situ collection, despite the significant inter-population differences noted in situ. The soil type and supply of soil organic matter, total nitrogen and other nutrients turned out to be most important for the quality of plants in terms of both their growth and biosynthetic capacity. In addition, in vitro micropropagation has proven to be a suitable method for accelerating seedling production. These results would be of practical importance in establishing an agricultural plantation of *G. glabra*.

Keywords: licorice; glycyrrhizin; total flavonoids; seed viability; ex situ propagation

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1. Introduction

Glycyrrhiza glabra L., commonly known as licorice, is a perennial herbaceous plant from the Fabaceae family. Its stems attain heights up to 120 cm, the leaves are multifoliate and imparipinnate, the flowers are violet, with axillary inflorescences (blooming in March), the fruits are compressed pods with several reniform seeds (fruiting in August), and the rhizomes and roots are stout [1]. The general distribution of *G. glabra* covers Southern Europe (Mediterranean), Eastern Europe and Southwest Asia [2], while in many places in Southwest Europe, the species is naturalized [3].

The numerous applications of *G. glabra* in the pharmaceutical, cosmetic and food industries are related mainly to the healing properties of the triterpene saponin glycyrrhizin and the flavonoids accumulated in the roots [1]. The traditional use of licorice as a remedy for ulcers and as a demulcent, expectorant, antitussive, laxative, and carminative dates back more than 4000 years; in modern medicine, licorice extracts are used to treat chronic hepatitis and as an antiviral agent against human immunodeficiency virus (HIV) and *Herpes simplex*, while deglycyrrhizinated preparations are applied to heal various types of ulcers, skin eruptions, psoriasis, and herpetic lesions [1]. Since the content of the main secondary metabolites significantly increases with age, the roots of 3-year-old and older plants are used in medical practice [4].

In a comparative study of 17 natural populations from different countries (Russia, Spain, France, the Netherlands, Germany, Iran, Afghanistan and China), a large difference in glycyrrhizin content was reported: between 0.99% and 7.01% [5]. Most of these populations do not meet the European Pharmacopoeia 7 [6] of a minimum of 4% glycyrrhizic acid in the dried drug, but contain glycyrrhizin above 2.5%, which is the requirement of the Japanese Pharmacopoeia 15 [7]. Similar variations in glycyrrhizin content from 1.36% to 3.40% were noted for 12 populations in Iran [8]. According to the authors, genetic diversity is the main reason for the significant variations in glycyrrhizin content, although rainfall, geographic location and altitude also influence its biosynthesis and accumulation in roots. The great importance of the genetic strains was also indicated after comparing the contents of glycyrrhizin and liquiritin in one hundred 5-year-old *G. uralensis* plants obtained from seeds and cultivated under the same field conditions [9]. Since the glycyrrhizin content of these plants varied between 0.46% and 4.67%, they selected the high-yielding plants and propagated them by stolon cuttings and by in vitro clonal propagation.

Overexploitation and lack of control over natural populations of *G. glabra* in Bulgaria led to their rapid decline due to the use of plant roots [10]. The authors presented data on the reduction of their areas over a period of 40 years: in 1957, the localities near the villages of Dolni Vit and Koilovtsi, Pleven district, occupied 14 dka each, and in 1996, 0.05 dka and 2 dka, respectively, while the locality close to Beltsov village, Ruse district, decreased from 12 dka to 2 dka. The most drastic depletion was reported for Nikopol locality, which decreased from 26 dka to 3 dka in the same period, and according to the latest data from RIEW-Pleven it has now disappeared (pers. communication). According to the IUCN categories and criteria, *G. glabra* has been assessed and categorized as endangered on the Red List of Bulgarian vascular plants [11]. Part of the *G. glabra* localities fall into protected areas of the European ecological network NATURA 2000 in Bulgaria. The species is protected by the Biological Diversity Act [12]. It is included in the Red Data Book of the Republic of Bulgaria as "endangered", and its cultivation is one of the recommended additional conservation measures [13]. To solve the problem of the shortage of *G. glabra* resources, the researchers proposed propagation of planting material starting from the best genotype [10]. The population near the village of Beltsov was assessed as the one with the highest content of glycyrrhizin [14]; however, licorice cultivation has not been implemented. Our recent analyses in all surviving populations, conducted by the more precise chromatographic method HPLC, confirmed that the Beltsov origin was the richest in glycyrrhizin ($p < 0.001$), with a value during the fruit-set stage, i.e., the harvest season, of 29.6 $\pm$ 2.3 mg/g DW [15]. To establish a plantation of economic importance, it is necessary to produce a huge quantity of seedlings in a short period of time, which could be realized by applying in vitro micropropagation, starting from a limited initial plant material (seeds, vegetative organs) [16,17]. Our previous study showed heavy microbial contamination of *G. glabra* stolons and relatively low seed germination [18], but no research on seed viability has been carried out so far.

The present study deals with the creation of an ex situ collection of *Glycyrrhiza glabra* using plant material originating from Bulgarian populations of the species and with the evaluation of the main characteristics of the cultivated plants in relation to their potential use as a source of plant material for the establishment of a plantation.

2. Materials and Methods

2.1. Plant Material and Soil Samples

Plant material was collected in October 2017 with the special permission of the Ministry of Environment and Waters (# 719/29.08.2017) from three Bulgarian populations of *G. glabra* near the villages of Dolni Vit (43.65861° N; 24.75902° E, Pleven district, 23 m a.s.l.), Koilovtsi (43.46009° N; 24.78099° E, Pleven district, 199 m a.s.l.) and Beltsov (43.59176° N; 25.60944° E, Russe district, 27 m a.s.l.). Stolon cuttings were used to obtain plants by vegetative propagation. Root segments from the same wild plants, and also from the 3-year-old plants grown in the experimental field plot of the Institute of Biodiversity and Ecosystem Research (IBER) in Sofia, were collected for analyses of their glycyrrhizin and total flavonoid contents. Seeds were collected from the same populations, along with root segments. Soil samples were taken from both wild populations near donor plants and the experimental field plot of IBER, from a depth of 30–50 cm. In addition, seeds and root segments from referent plants originating from a commercial plantation in Ukraine were used for comparative analyses. In vitro propagated plants obtained as previously described [18] were used for ex vitro adaptation and outdoor acclimatization.

2.2. In Vivo Vegetative Propagation

Stolon cuttings collected from the wild donor plants in autumn were immediately planted in the IBER ex situ collection, 550 m above sea level. The emergence of new plants and their growth and development were monitored over several years.

2.3. Seed Viability

To assess seed viability, a rapid 24 h tetrazolium test was applied according to Peters [19] on 100 seeds per population, and the interpretation of the results was conducted following Moore [20].

2.4. Acclimatization of In Vitro Propagated Plants

A total of 185 well-shaped in vitro plants from all origins tested were potted in a substrate mixture containing light-mix soil, coconut fibers, and sand in a 2:1:1 proportion and ex vitro adapted into a growth camera (POL-EKO-APARATURA KK350) for 6 to 8 weeks under strict control of the temperature, light, and relative air humidity, as previously described for other species [21]. Plants were then put on the shelves in a room phytotron for several months for additional strengthening at temperature of 25 $\pm$ 3 °C, mixed sun and artificial LED light for 16 h daily, and relative air humidity between 30 and 50%. After transplanting into larger pots, they were transferred to an unheated greenhouse. Several plants originating from different populations were acclimated outdoor in the experimental field plot of IBER.

2.5. Phytochemical Analyses

Glycyrrhizin and total flavonoids in methanol root extracts of donor wild-growing plants and 3-year-old cultivated plants were determined using HPLC and spectrophotometry, respectively, as previously described [15].

2.6. Statistical Analyses

The differences between the contents of glycyrrhizin and total flavonoids in the roots of in situ grown and ex situ cultivated *G. glabra* plants were analyzed by Excel ANOVA single factor.

2.7. Soil Analyses

The acid/base reaction pH of the air-dry soil samples was measured according to ISO 10390, 1:5 (*w:v*), Soil quality: Determination of pH, https://www.iso.org/standard/40879.html (accessed on 7 December 2023). Soil samples were analyzed by Nik Agro Service Ltd. for their physical and chemical characteristics: particle size, soil organic matter (SOM), total nitrogen, and plant available phosphorus, potassium and calcium, and carbonates (according to ISO 11277:2020, Soil quality: Determination of particle size distribution in mineral soil material, https://www.iso.org/standard/69496.html (accessed on 9 January 2024), ISO 13878:1998 Soil quality: Determination of total nitrogen content by dry combustion ("elemental analysis"), https://www.iso.org/standard/23117.html (accessed on 9 January 2024) and [22].

3. Results

3.1. Establishing an Ex Situ Collection by Vegetative Propagation via Root Cuttings

Stolon segments collected from three Bulgarian populations (Dolni Vit, Koilovtsi, and Beltsov) were planted in autumn 2017, and the first shoots appeared in the spring of the following year (Figure 1A). Already in the first growing season, differences between the individuals originating from the different localities are noticeable, with those originating from Dolni Vit forming the largest plant mass and more and taller shoots than the others. This trend continued in the following years (Figure 1B). Under the conditions of ex situ cultivation, the plants preserved their natural characteristics, that is, the shoots dried up every autumn, and new ones appeared in the spring. The plants reached maturity after two years, producing some inflorescences and few seeds. However, their vegetative propagation was quick. A tendency was observed of rapid expansion of the area occupied by *G. glabra* owing to the formation of underground stolons in all directions and the emergence of shoots several meters from the initially planted root cuttings (Figure 1C). Plants originating from the Dolni Vit locality produced multiple stolons and their shoots appeared approximately 5 m from the initial plant.

Figure 1. Ex situ collection of *G. glabra* originating from three Bulgarian populations: (**A**) First shoots rising in the spring; (**B**) 3-year-old plants; (**C**) Expansion of plants (initial plants are marked with red ellipses and new shoots with yellow ellipses).

3.2. Establishing an Ex Situ Collection by In Vitro Propagation, Starting from Seeds

The second part of the ex situ collection includes in vitro multiplied plants starting from seeds from the same populations and from a commercial Ukrainian plantation as referent origin. Seed disinfection and germination were carried out as previously described [18]. It took 6 months from the initiation of the in vitro culture to the regeneration of whole rooted in vitro plants after the first subcultivation. The in vitro obtained plants were multiplied with several consecutive subcultivations on the already selected medium both for a relatively high propagation coefficient and for the best plant quality: MS based medium [23] containing 30 g/L sucrose, supplemented with plant growth regulators (1.0 mg/L kinetin and 0.5 mg/L indole-3-butyric acid) and solidified with 6.5 g/L plant agar (Duchefa, NL). The first step of the ex vitro adaptation was the most difficult (Table 1). A total of 185 in vitro plants from all origins, approximately 10 cm in height, were potted in a soil mixture and transferred into the growth camera, where over half of them died, although the others were well growing and some of them ramified (Figure 2A). The plants needed 6 to 8 weeks to strengthen. The second step of the ex vitro adaptation, in a room phytotron, was more successful (Table 1, Figure 2B). In 2019, almost all plants were successfully acclimatized in an unheated greenhouse and reached approximately 80 cm in height, which was limited by the size of the pots (Table 1, Figure 2C). Two plants per origin were transferred to the experimental field plot of IBER in April 2019 and were successfully acclimatized outdoors (Figure 2D). However, their growth was slower than the plants derived from stolon cuttings. Among the ex vitro acclimated plants, those originating from the Ukrainian plantation developed faster than the other ones.

Table 1. Ex vitro adaptation of in vitro regenerated *G. glabra* plants.

Origin	Growth Camera			Room Phytotron		Greenhouse	
	Initial Number	Surviving		Surviving		Surviving	
		Number	%	Number	%	Number	%
Dolni Vit	13	7	53.8	7	100.0	7	100.0
Koilovtsi	57	17	29.8	9	52.9	7	77.8
Beltsov	5	5	100.0	4	80.0	4	100.0
Ukraine	110	49	44.5	29	59.2	25	86.2
Total	185	78	42.2	49	62.8	43	87.8

Figure 2. Ex situ collection of *G. glabra* obtained from in vitro propagated plants starting from seeds: ex vitro adaptation in the growth camera (**A**) and in the room phytotron (**B**); Acclimatization in the greenhouse (**C**) and outdoors in the experimental field plot (**D**).

3.3. Seed Viability

As a result of the staining of the embryos after applying the viability test, the seeds were grouped into 3 classes depending on the degree of their staining by the tetrazolium solution: Class I—embryos stained red, Class II—embryos stained pink, and Class III—unstained embryos. Germless seeds were also found, which were separated into a fourth class: Class IV—empty seeds. The qualitative assessment and the percentage ratio of the seeds of the different classes are presented in Figures 3 and 4. According to the criteria for interpreting the results of the Tetrazolium test [20], Class I and Class II seeds were assessed as viable, i.e., viable seeds were 48% for the Dolni Vit population, 46% for the Koilovtsi population and 40% for the Beltsov population.

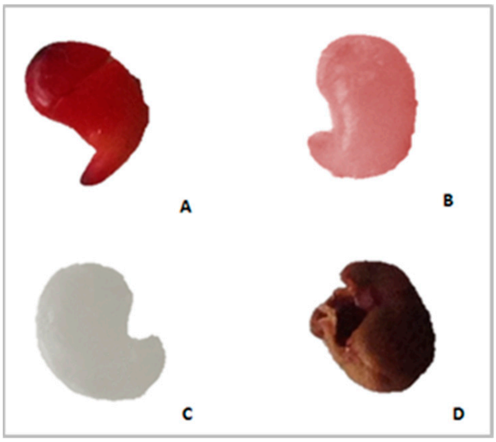

Figure 3. Qualitative assessment of *G. glabra* seed viability according to the tetrazolium test. Viable seeds: seed stained red (**A**) and stained pink (**B**); unviable seeds: unstained seed (**C**) and empty seed (**D**).

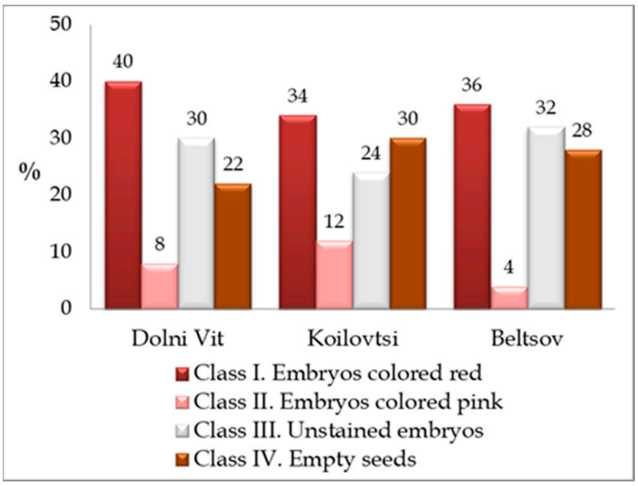

Figure 4. Quantitative assessment of the viability of *G. glabra* seeds collected from 3 Bulgarian populations (viable seeds are stained red or pink; unviable seeds are unstained or empty).

3.4. Glycyrrhizin and Total Flavonoid Contents in Plants Growing In Situ and Ex Situ

Comparative analyses of glycyrrhizin and total flavonoid contents were performed for the in situ grown and 3-year-old ex situ cultivated plants, the latter originating from wild plants. The contents of both glycyrrhizin and total flavonoids in the in situ grown and ex situ cultivated plants were similar for the Koilovtsi and Beltsov origins, while those for the Dolni Vit origin differed considerably due to their increase under the control conditions (Figures 5 and 6), which were statistically proven: $p < 0.01$ for the glycyrrhizin content (Table 2) and $p < 0.001$ for the total flavonoid content (Table 3).

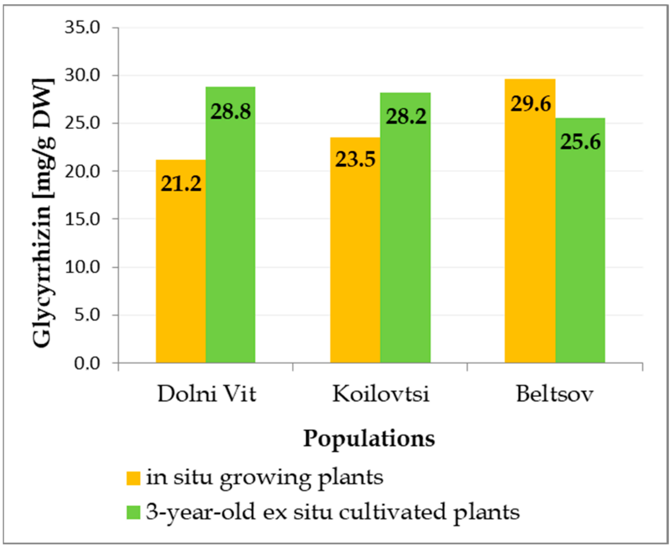

Figure 5. Glycyrrhizin content in the roots of in situ grown *G. glabra* plants and in 3-year-old ex situ cultivated plants originating from three Bulgarian populations.

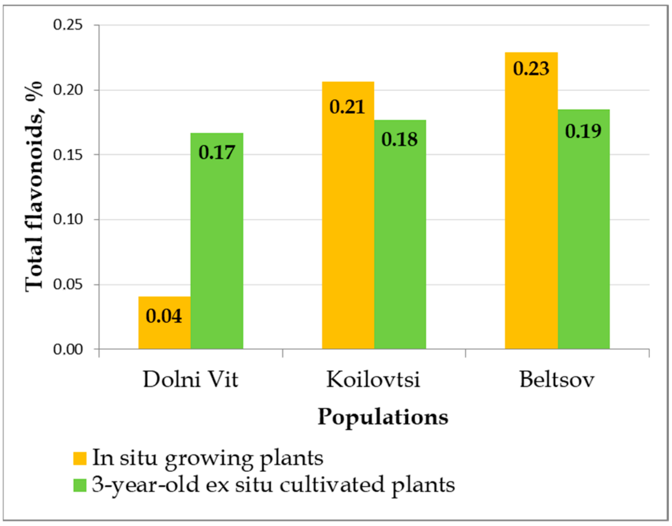

Figure 6. Total flavonoid content in the roots of in situ grown *G. glabra* plants and in 3-year-old ex situ cultivated plants originating from three Bulgarian populations.

Table 2. Difference between glycyrrhizin content in roots of in situ grown and 3-year-old ex situ cultivated *G. glabra* plants originating from Dolni Vit (Excel ANOVA single factor).

Summary						
Groups	**Count**	**Sum**	**Average**	**Variance**		
in situ 2017	3	63.53309	21.1777	1.271251		
ex situ 2020	3	86.47017	28.82339	3.063705		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	***p*-Value**	**F crit**
Between Groups	87.68495	1	87.68495	40.45483	0.003132	7.708647
Within Groups	8.669911	4	2.167478			
Total	96.35486	5				

Table 3. Difference between total flavonoid content in the roots of in situ grown and 3-year-old ex situ cultivated *G. glabra* plants originating from Dolni Vit (Excel ANOVA single factor).

Anova: Single Factor						
Summary						
Groups	**Count**	**Sum**	**Average**	**Variance**		
in situ 2017	4	0.151	0.03775	0.000604		
ex situ 2020	3	0.502	0.167333	0.000142		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	***p*-Value**	**F crit**
Between Groups	0.028786	1	0.028786	68.68804	0.000417	6.607891
Within Groups	0.002095	5	0.000419			
Total	0.030881	6				

3.5. Soil Characteristics

The main physical and chemical soil characteristics of the samples taken from the wild Bulgarian *G. glabra* populations and the experimental field plot are presented in Table 4. All the investigated soils ranged from slightly acidic to slightly alkaline: 7.51 for Dolni Vit, 7.38 for Beltsov, 6.88 for Koilovtsi, and 6.76 for the experimental field plot. The percentages of the carbonates varied considerably, the highest one noted for Beltsov, 9.02%, followed by those of Dolni Vit, 4.34%, the field plot, 0.95%, and Koilovtsi, 0.40%.

Table 4. Characteristics of the soil samples taken from the wild Bulgarian *G. glabra* populations and the experimental field plot.

(A) Physical Soil Characteristics					
Soil Sample	**Particle Size Distribution**				
	2–0.2 mm	**0.2–0.02 mm**	**0.02–0.002 mm**	**<0.002 mm**	**<0.01 mm**
Dolni Vit	20.90%	67.10%	8.00%	4.00%	8.80%
Koilovtsi	4.90%	39.70%	22.80%	32.60%	43.60%
Beltsov	4.20%	45.10%	24.10%	26.60%	38.60%
Field plot	39.70%	23.50%	17.70%	19.10%	29.70%
(B) Chemical Soil Characteristics					
Soil Sample	**Total N %**	**P_2O_5 mg/kg**	**K_2O mg/kg**	**CaO mg/kg**	**SOM %**
Dolni Vit	0.03	214.61	70.40	10,371.46	1.71
Koilovtsi	0.23	2460.98	269.28	7378.39	4.93
Beltsov	0.20	1058.02	278.51	11,024.85	3.37
Field plot	0.15	165.42	156.64	5651.90	4.86

4. Discussion

The establishment of an ex situ collection of *Glycyrrhiza glabra* allowed the evaluation of the behaviors of plants with different origins cultivated under the same controlled conditions, in terms of plant growth and development, as well as the accumulation of the secondary metabolites of interest. This research was necessary before the creation of a plantation, as the yield of the bioactive substances depends on both plant genetic features and soil characteristics. Such a comparative in situ–ex situ study of *G. glabra* plants originating from the Bulgarian populations has not been conducted so far.

For seed propagation to become applicable in commercial licorice cultivation, a drastic increase in seed germination is required [24]. Some authors succeeded in overcoming seed dormancy by using chemical scarification with sulfuric acid solutions and achieved over 90% germinated seeds [25]. In our case, the seed germination rate was limited by the relatively low seed viability, which varied between 40% and 48% for the three Bulgarian populations; in addition, approximately a quarter of all the seeds were empty (Figure 4). The percentage of germinated seeds determined in our previous studies [18] corresponded to that of the viable seeds.

On the other hand, the conventional propagation of *G. glabra* by stolon division was also reported to be slow and inefficient [26]. In order to provide planting material for the cultivation of the species to meet the needs of the pharmaceutical industry, the use of in vitro techniques for accelerated breeding was recommended [17]. In spite of the heavy microbial contamination, in vitro cultures were initiated using internodes and apical or axillar buds as primary explants, and plants were regenerated directly or through calluses [16,17,26,27].

The success of ex vitro adaptation may depend on the plant species; for example, in previous studies in our laboratory, under the same conditions, almost all in vitro propagated plants of *Centaurea davidovii* and *Arnica montana* survived in the growth camera [28,29], while only a few *Papaver degenii* plants adapted successfully, despite their well-developed roots [30]. The success of ex vitro adaptation of *G. glabra* was found to depend strongly on the composition of the soil substrate, with survival rates varying between 11.1% and 77.7% [26]. The authors stated that the differences between the eight substrate mixtures tested were due to their physical characteristics, and the best growth medium contained clay soil and peat moss, providing nutrient availability and water-holding capacity (1:1 v/v). The soil substrate used in our experiment ensured both water retention and aeration of the root zone owing to the coconut fibers and sand component, respectively. However, the first stage of ex vitro adaptation needs to be improved, probably by optimizing the ratio of the substrate components.

Since the content of glycyrrhizin in *G. glabra* roots was proven to depend on both genetic diversity and environmental conditions [8,9], it was important to select plants with high glycyrrhizin concentration as a starting material and to determine the most suitable soil type for the creation of a plantation. Among the Bulgarian populations, only the one near the village of Beltsov contains glycyrrhizin above 2.5%, which meets the requirements of the Japanese Pharmacopoeia 15 [7]. However, under the conditions of the ex situ collection, the 3-year-old plants from all investigated origins produced glycyrrhizin in higher concentrations (Figure 5), which suggested insignificant genetic variation in the natural Bulgarian populations. This is consistent with the hypothesis of a common origin of all Bulgarian licorice populations in the Danube Plain, based on the memories of elderly local people, according to whom the species was introduced from Türkiye (pers. comm.). Our observations showed that the populations are compact, with predominant vegetative propagation through stolons, which connect the plants in a large underground network. Taking into account their very small size, between 0.005 and 0.3 ha [10], and the findings of some authors [31] that within-population genetic diversity of *G. glabra* correlated with the population size, the genetic variation of the Bulgarian populations is expected to be very low. Future studies of the genetic diversity of the Bulgarian populations of *G. glabra* could clarify this assumption.

On the other hand, in studies by the Shaanxi Institute of Sand Control in China aimed at developing new areas for agriculture, it was shown that both licorice root yield and glycyrrhizin content depended on soil nutrients [32]. Cultivation of *G. glabra* was carried out in the experimental field on sand as a control and three other soil substrates: sand enriched with biochar, with soft rocks, or with a combination of both. The results showed the lowest yield and lowest glycyrrhizin content in the sand control and the highest values in the biochar-enriched sand. Data from our soil analyses (Table 4) confirmed these outcomes. According to the known classifications, only the soil from Dolni Vit has a low supply of SOM (humus) [33] and a very low supply of total nitrogen [34]. Regarding the plant available potassium, the soils from the Beltsov and Koilovtsi localities are very well supplied, that near Dolni Vit village is moderately supplied [34], and the supply of the experimental field soil is intermediate. Thus, the sharp increase in the contents of glycyrrhizin and the total flavonoids observed in the ex situ cultivated plants of Dolni

Vit origin (Figures 5 and 6, Tables 2 and 3) can be explained by the significant differences between the soil characteristics of this locality and those of the experimental field. In the other two origins, the differences in the contents of the secondary metabolites of interest are statistically insignificant, which corresponds to the similar values of SOM and total nitrogen in situ and ex situ (Table 4).

Actually, the data from our soil analyses differ from the expected ones, because according to the soil map of Bulgaria [35], the localities of *G. glabra* near the villages of Dolni Vit and Beltsov fall within the distribution zone of soils of the Fluvisol class (alluvial), which are genetically young, and that close to Koilovtsi village falls in the zone of the most fertile soils of the Chernozems class (soil names are according to the World Reference Base for Soil Resources) [36]. Licorice localities, however, occupy very small areas, which may explain the differences in the soil characteristics near Dolni Vit. It was reported [37] that both localities, Dolni Vit and Beltsov, were characterized by high soil porosity, which was associated with good water permeability and did not allow the accumulation of water reserves during spring rains. The authors stated that during prolonged periods of drought, sandy soils with high porosity cannot provide sufficient amounts of plant available nitrogen to support plant assimilation. Indeed, we found that the soil in the locality of *G. glabra* near Dolni Vit village was sandy, with erosion in places, but the *G. glabra* population near Beltsov village was situated in a narrow strip between two fields, slightly lower than them, with a lot of bushes and trees, which did not allow the soil to dry out. The soil analyses confirmed the differences between the localities: only in the sample from the Dolni Vit locality did the large soil particles (2 to 0.02 mm) predominate, and the percentage of SOM was 1.71%, i.e., significantly lower than in the other soil samples (Table 4). Under the controlled conditions of the ex situ collection, with the application of regular watering and weeding, a rapid growth of the plants and development of numerous stolons and shoots were noticed, especially concerning plants originating from the Dolni Vit locality.

According to some authors, licorice has good adaptive properties in terms of average annual air temperature (5–25 °C), annual rainfall (between 400 and 1160 mm) and soil pH (5.7–8.2) and prefers deep and well-drained site soils, such as sandy soils, which are suitable for its normal growth, but it also develops well on rich chernozems [24]. All soil samples ranged from slightly acidic to slightly alkaline, which is appropriate for the species [24] and is consistent with the data of other authors for the soils of these classes [33]. Some authors stated that rainfall, geographic location and altitude also affect glycyrrhizin biosynthesis, although genetic diversity is the main reason for the significant variation in its content [8]. The altitude of the ex situ collection is 550 m, which is higher compared to all Bulgarian *G. glabra* localities, but this did not affect the plants in terms of their growth and the content of their main secondary metabolites.

The first comparative study of in situ grown and ex situ cultivated *G. glabra* plants originating from the Bulgarian localities of the species showed that the contents of glycyrrhizin and total flavonoids depended mostly on the soil characteristics. Despite the fact that the population near the Beltsov village is significantly richer in glycyrrhizin compared to the other Bulgarian *G. glabra* populations, all origins are suitable as a source of starting plant material, as the contents of the main secondary metabolites leveled off under the ex situ cultivation conditions. In terms of plant mass yield, the most productive were the ex situ cultivated plants originating from the population of Dolni Vit. Our findings led to a revision of the previous recommendation to establish a field plantation with plant material originating from the Beltsov population. Soil type and soil nutrients, especially the soil organic matter content and total nitrogen, turned out to be crucial for the biosynthesis of the secondary metabolites of interest. In vitro micropropagation would be a useful tool to accelerate the seedling production needed to create a plantation. However, to preserve the genotype of the donor plants and avoid undesired changes in glycyrrhizin and total flavonoid contents, in vitro cultures should be initiated from vegetative organs such as internodes and buds, following existing protocols in the literature.

5. Conclusions

The results of our study would be of practical importance in establishing an agricultural plantation of *G. glabra*. We found out that under the controlled conditions of an ex situ collection, plants originating from all known Bulgarian populations of *G. glabra* produced glycyrrhizin and flavonoids in similar concentrations, despite the inter-population differences noted in situ. The type of soil and its supply of soil organic matter, total nitrogen, and other nutrients proved to be most important for the quality of the plants in terms of both their growth and the biosynthesis of the main secondary metabolites. Plants originating from the Dolni Vit population appeared to be the fastest growing and therefore the most appropriate as a source of plant material for plant production. In vitro micropropagation has proven to be a suitable method for accelerated production of *G. glabra* plants. Our future studies will focus on several points: in vitro clonal propagation of *G. glabra* using vegetative organs as primary explants, taken from ex situ cultivated plants, in order to keep the selected genotype; monitoring glycyrrhizin and total flavonoid content after longer ex situ cultivation of plants in the collection to check possible fluctuations in their levels; and determination of the content of the main secondary metabolites in the roots of in vitro propagated and outdoor acclimatized plants.

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