

Article

In Vitro Plantlet Regeneration and Accumulation of Phenolic Compounds in Microshoots of *Astragalus glycyphyllos* L.

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Abstract

Astragalus glycyphyllos (Fabaceae) is known to be a source of flavones, flavonols, and isoflavones, and its in vitro culture may promote the efficiency and sustainability of obtaining pharmacologically valuable fractions. The aim of this study was to develop an effective plantlet regeneration protocol for *A. glycyphyllos*, providing the accumulation of phenolic compounds and antioxidants in cultured tissues. The results show a maximum seed germination rate (67.8%) after scarification (mechanical with sandpaper followed by treatment with 50% sulfuric acid) and subsequent sterilization with 1.1% sodium hypochlorite solution. The maximum regeneration rate (95%) was achieved on Murashige and Skoog medium supplemented with 0.5 mg·L⁻¹ thidiazuron. A thidiazuron concentration of 0.05 mg·L⁻¹, combined with a twofold increase in iron chelate content, induced the maximum yield of total flavonoids (8.74 mg·g⁻¹ DW), and significant levels of total phenolics (4.15 mg·g⁻¹) and antioxidants (1.83 mg AAE·g⁻¹) in the microshoot tissues. HPLC analysis showed kaempferol glycosides (1.51 mg·g⁻¹) and acylated kaempferol glycosides (2.76 mg·g⁻¹) as major components. Formononetin in a modest amount (0.09 mg·g⁻¹) was detected in hydrolyzed extracts. The phenolic profiles of the microshoots and native plants coincided in hydroxycinnamic acid composition; meanwhile, quercetin glycosides were present only in situ plants, and formononetin was found only in the plantlets. The results confirm the prospects of biotechnological methods for the industrial production of standardized medicinal raw materials.

Keywords: *Astragalus glycyphyllos*; seed dormancy; plant growth regulators; thidiazuron; iron chelate; phenolic compounds; kaempferol; in vitro-derived plants



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1. Introduction

Discovering new plant sources of pharmacologically active compounds is increasingly in the focus of contemporary research. The development of novel plant-derived medicines with low toxicity, high efficacy, good patient tolerance, and minimal side effects has become a global priority. Their relevance continues to rise in the face of the rapid spread of human diseases [1,2]. Wild plant species are a valuable biological resource and represent an important part of the raw material potential of countries. Many of them are rare and endangered plants, and their use is restricted due to the risks associated with overexploitation, damage to their natural habitats, and uncontrolled human activities [3]. One way to conserve and expand the bioresource base and take advantage of the economically valuable features of new non-traditional plants is the development of technologies for creating in vitro cultures

of resource species and establishing artificial ecosystems. This approach will help avoid the removal of biological objects from natural populations for their cultivation [4–6].

The demand for plant-based raw materials is huge all over the world. However, humanity is now coming to realize that it is impossible to meet the needs of the market solely by harvesting wild plants. In addition, the standardization of plant-derived drugs presents a particular problem, since their composition and the content of valuable metabolites vary significantly depending on environmental conditions, climatic changes, infection by microorganisms, the effects of pesticides, insect infestations, production methods, etc. Meanwhile, the chemical synthesis of target secondary metabolites is difficult due to the complexity of their structures and stereospecificity [7]. Biotechnological approaches, including the development of systems for cultivating medicinal plants in vitro, represent an attractive alternative for the production of pharmacologically significant compounds [8].

Various in vitro cultivation strategies (elicitation, hairy root cultures, suspension cultures, etc.) are widely used to improve the production of plant secondary metabolites. In vitro technologies allow for the production of biomass regardless of season, climate, or soil conditions. They also facilitate the extraction and purification of target compounds. Totipotency is the property that enables isolated plant cells to produce a variety of secondary metabolites in vitro, which are typically synthesized in vivo by plants. This genetic potential can be realized through specific cultivation strategies to enhance the yield of valuable compounds [9].

The presented study is a continuation of our series of studies dealing with *Astragalus* tissue culture and their phenolic compounds [10,11]. The in vitro-derived plants of *A. sericeocanus* showed a rich aglycone composition (7 compounds), while the concentrations of most aglycones, including quercetin, were not high ($0.1\text{--}0.2\text{ mg}\cdot\text{g}^{-1}\text{ DW}$). Isorhamnetin was the predominant aglycone ($0.13\text{ mg}\cdot\text{g}^{-1}\text{ DW}$), and kaempferol was also found in moderate quantities ($0.04\text{ mg}\cdot\text{g}^{-1}\text{ DW}$). The complete aglycone composition was detected only in generatively grown in vivo plants; meanwhile, quercetin and luteolin were not found in the seedlings [12]. In addition, we induced and established an *A. penduliflorus* hairy root culture by co-cultivating seedling explants with *Rhizobium rhizogenes*. The hairy roots showed total phenolic compounds of up to $6.3\text{ mg}\cdot\text{g}^{-1}\text{ DW}$, with hydroxybenzoic acids (including gallic acid) and flavonoids such as baicalin and isoliquiritigenin as major compounds. Extracts of the roots manifested pronounced radical scavenging and anti-influenza activity [13]. Thus, in vitro culture of *Astragalus* plants can be considered a sustainable source of active metabolites.

Astragalus is a large genus of perennial herbaceous plants, comprising approximately 2500 species. The most well-known and medicinally important species, possessing the widest spectrum of pharmacological properties, are *A. dasyanthus*, *A. adsurgens*, *A. virgatus*, *A. membranaceus*, *A. galactites*, *A. galegiformis*, *A. onobrychis*, *A. mongholicus*, *A. vulpinus*, *A. zingeri*, *A. falcatus*, *A. glycyphyllos*, and many others [14]. The therapeutic properties of a number of *Astragalus* species are mainly attributed to saponins, flavonoids, and polysaccharides [15]. Medications based on aqueous, ethanolic, and other organic solvent extracts of their above-ground and underground parts have been shown to exert a wide array of biological effects, in particular, immunomodulatory, antioxidant, antitumor, antibacterial, antiviral, cardiotonic, hepatoprotective, and antidiabetic activities [15,16]. In Russia, only *A. dasyanthus* and *A. falcatus* have been incorporated into the Russian State Pharmacopoeia. The medication Flaronin, which has hypoazotemic and diuretic action, based on *A. falcatus* flavonoids, has been developed [17]. Similar to *A. dasyanthus*, which exhibits a variety of pharmacological properties [18], *A. glycyphyllos* has also shown therapeutic potential [19–21]. Since *A. dasyanthus* has not been found in Siberia [22], *A. glycyphyllos* is of interest as a source of health-promoting metabolites.

A. glycyphyllos was reported to contain biologically active compounds of various classes: phenolic compounds, terpenoids, alkaloids, higher fatty acids, amino acids, and polysaccharides [23–31]. Phenolic compounds of *A. glycyphyllos* are represented by hydroxycinnamic and hydroxybenzoic acids, flavonoids, and isoflavonoids. Glycosides of flavonols (kaempferol, quercetin, and isorhamnetin) and flavones (such as apigenin glycosides) have been shown to be the main flavonoid constituents of *Astragalus*. Previously, the following flavonoid glycosides in the aboveground part of *A. glycyphyllos* were identified: cosmosiin, astragalin, isorhamnetin-3-O- β -diglucoside, apigenin-7-O-arabinosylglucoside, kaempferol-3-O-xylosylglucoside, kaempferol-7-O-galactoside, rutin, camelliaside A, and mauritianin [24,26,29,32]. Four isoflavones have been identified in the leaves, stems, and flowers of *A. glycyphyllos*: biochanin A, daidzein, formononetin, and genistein [23,33]. Chlorogenic, caffeic, *p*-coumaric, ferulic, and rosmarinic acids [34], (–)-gallocatechin, (–)-epigallocatechin, (+)-catechin, (–)-epicatechin, (–)-epicatechin gallate, and gallic acid were identified and quantified in the aboveground part of *Astragalus* plants [35].

A. glycyphyllos is widely used in the traditional medicine of many countries in Europe and Asia (France, Bulgaria, Ukraine, Belarus, Russia) as an emollient, diuretic, antihypertensive, and anti-inflammatory agent [36]. Hypotensive, anticoagulant, diuretic, antimicrobial, antioxidant, and neuroprotective activities have been found in biochemical assays and clinical trials [19,27]. In Bulgaria, a dry tablet extract was obtained from the aboveground part of *A. glycyphyllos* with a pronounced antiviral effect against human alphaherpesvirus 1 (including acyclovir-sensitive strains) standardized in terms of saponin content [37].

Currently, *A. glycyphyllos* is primarily harvested from wild populations and is utilized in medicine for its medicinal properties or as a forage plant. Established large-scale agricultural protocols for this species are absent. Therefore, the second assignment of tissue culture is to be an alternative source of biochemicals, including phenolic acids, flavonols, and isoflavonoids. To date, despite the widespread use of *A. glycyphyllos* in the folk medicine of many countries, reports on its in vitro propagation are rare [38,39].

Methods of clonal micropropagation of *Astragalus* species are extensively developed to maintain the stability of native populations [40,41]. Furthermore, the capacity of tissue culture to enhance phenolic metabolite production in this plant is far from exhausted. In this context, the development of an integrated protocol that combines efficient regeneration with the stimulation of secondary metabolism is of high priority for pharmaceutical applications. Information on increasing in vitro biosynthesis of bioactive compounds is still scarce. Investigations in this field deal with species endemic to Mediterranean regions (*A. aitosensis*, *A. thraciensis*, *A. fruticosus*, *A. gymolobus*, *A. hamosus*, *A. chrysochlorus*) and North America (*A. missouriensis*) [42–46]. We can find only one study concerned with phenolic metabolites of the widespread, cold-tolerant species *A. glycyphyllos*, whose range encompasses most of Europe, Central and Western Asia, including Western Siberia [38]. The value of these plants is in specialized metabolites involved in physiological and biochemical support of cold stress resistance through scavenging ROS and membrane stabilization [47]. Meanwhile, the concentration of these stress-responsive metabolites usually decreases in controlled conditions of tissue culture [48]. Accordingly, it is of interest to examine a tissue culture of *A. glycyphyllos* from Western Siberia as a source of phenolic compounds. Our previous results showed the abundance of glycosides of quercetin, kaempferol, and isorhamnetin in *Astragalus* species growing in Siberian conditions [10,49].

In our earlier investigations and in other authors' studies, various techniques (light regime, Ca²⁺ and Mg²⁺ supplement, elicitors) have been applied to enhance the yield of target compounds [38,46,50]. Yeast extract, sucrose, casein, methyl jasmonate, salicylic acid, quercetin, and a complex plant-derived supplement were used as elicitors [38,43,46,48,51].

To our knowledge, no studies have developed a reproducible protocol for in vitro culture establishment of *A. glycyphyllos* or obtained in vitro-derived tissues with enhanced phenolic compound production and antioxidant activity. Therefore, in the present study, our objectives were (a) to develop a seed germination protocol; (b) to assess the in vitro regenerative potential of plantlets depending on the combinations and concentrations of plant growth regulators and the type of nutrient medium; (c) to measure the content of the main photosynthetic pigments in microshoots; (d) to quantify the concentrations of phenolic compounds and flavonoids in the extracts from in vitro-derived plants and analyze them comparatively; and (e) to evaluate the antioxidant activity of the plant extracts.

2. Materials and Methods

2.1. Plant Material

The plants of *A. glycyphyllos* were identified and verified by the Department of Systematics of vascular plants at the Central Siberian Botanical Garden, Siberian Branch of the Russian Academy of Sciences (CSBG SB RAS), Novosibirsk, Russian Federation. A voucher specimen (accession number NS0063037) was deposited in the digital herbarium of the CSBG SB RAS and is available at <http://herb.csbg.nsc.ru:8081> (accessed on 22 March 2026). Mature seeds of *A. glycyphyllos* were collected in August 2022 from a natural population in Novosibirsk (geographical coordinates: 54°50'35.4" N 83°06'11.6" E). The seeds were harvested at the full fruit maturity stage. The seeds were stored at 5 °C for one year prior to the experiments. To overcome seed dormancy, four scarification treatments were tested: (1) mechanical scarification with sandpaper; (2) chemical scarification with 50% sulfuric acid (40 min) [12]; (3) combined mechanical scarification followed by treatment with 50% sulfuric acid; and (4) mechanical scarification followed by thermal treatment in water at 50 °C.

Following scarification, the seeds were surface-sterilized using one of two methods: (i) immersion in 70% aqueous ethanol for 2–3 min, followed by treatment with 37.7% hydrogen peroxide for 10 min, or (ii) immersion in 70% aqueous ethanol for 2–3 min, followed by treatment with 1.1% sodium hypochlorite solution for 25 min. In both cases, each sterilization step was followed by three rinses in sterile distilled water (10 min per rinse). The number of seeds for each variant was 200.

2.2. Seed Germination

Seeds were germinated on hormone-free Murashige and Skoog (MS) medium [52]. Germinated seeds were counted daily. The time of root emergence was recorded. The total germination percentage (GP) and average seed germination rate (Mdays) were calculated. A seed was considered germinated when the primary root exceeded 2 mm in length [53]. The total percentage of seed germination was determined using the following formula:

$$GP = (n_i/N) \times 100$$

where n_i is the number of germinated seeds at the end of the experiment, and N is the total number of seeds.

The mean seed germination time (the conventional number of days required for one seed to germinate) was calculated according to the M.A. Ranal and D.G. Santana method [54]:

$$M_{\text{days}} = \sum_{i=1}^n N_i \times G_i / \sum_{i=1}^n G_i$$

where N_i is the number of days from the beginning of cultivation to the i -th observation, and G_i is the number of seeds that germinated at the i -th observation.

2.3. In Vitro Shoot Organogenesis

To induce in vitro microshoot formation, a 2-mm stem segment with a single axillary bud, excised from 30-day-old plantlets, was used as an explant. Explants were cultured on standard MS medium and on a modified MS medium with a twofold concentration of iron chelate (MS with $2 \times$ Fe-chelate), supplemented with $30 \text{ g} \cdot \text{L}^{-1}$ sucrose and $6 \text{ g} \cdot \text{L}^{-1}$ Bactoagar (PanReac®, Barcelona, Spain). The media were further supplemented with plant growth regulators (PGRs) in various concentrations and combinations: (1) 1.0, 2.0, or $5.0 \text{ mg} \cdot \text{L}^{-1}$ 6-(2-isopentenyl)adenine (2-iP) combined with $0.1 \text{ mg} \cdot \text{L}^{-1}$ indole-3-acetic acid (IAA); (2) 1.0, 2.0, or $5.0 \text{ mg} \cdot \text{L}^{-1}$ 6-benzylaminopurine (6-BAP) combined with $0.1 \text{ mg} \cdot \text{L}^{-1}$ IAA (ICN Biomedicals, Aurora, OH, USA); or (3) 0.1, 0.5, or $0.05 \text{ mg} \cdot \text{L}^{-1}$ thidiazuron (TDZ) (Sigma Aldrich Laborchemikalien GmbH, Seelze, Germany). MS and MS with $2 \times$ Fe-chelate media without PGRs were used as controls. The pH of all media was adjusted to 5.7–5.8 prior to autoclaving at 121°C and $1.05 \text{ kg} \cdot \text{cm}^{-2}$. PGRs were added to the cooled media after autoclaving. Cultures were maintained in 200-mL glass jars containing 20 mL medium, and incubated at $23 \pm 2^\circ\text{C}$ under a 16-h photoperiod with a light intensity of $40 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ provided by cool-white fluorescent lamps for 8 weeks (Philips, Pila, Poland). The experiment was conducted twice with 10 explants per replicate, resulting in a total of 20 explants per treatment.

The axillary microshoot proliferation frequency—the percentage of explants producing axillary microshoots out of the total number of explants (%)—the number of microshoots per explant, the length of microshoots (cm), the number of internodes per microshoot, and the fresh weight (FW) of the microshoots (g) were measured. The dry weight (DW) of microshoots was measured after drying at 50°C for 48 h.

2.4. Extraction of Metabolites

The tissues of aboveground parts of 10 microshoots were combined in each sample for the spectrophotometric and high-performance liquid chromatography (HPLC) analyses and estimation of antioxidant activity. Determination of photosynthetic pigments was performed in samples of fresh microshoot tissues. To determine the total phenolic and flavonoid concentrations, air-dried leaves and stems (0.01 g) were homogenized with 1.5 mL of 95% ethanol. Phenolic compounds were extracted for 45 min at 45°C with periodic stirring. The extract was then centrifuged using an Eppendorf 5430 R centrifuge (Eppendorf®, Hamburg, Germany) at 16,000 rpm for 2 min. The supernatant was separated and used for the analysis.

For the HPLC analysis and determination of total antioxidant activity, the air-dried microshoots were powdered, weighed (0.2 g), and extracted twice. First, maceration was carried out with an aqueous-ethanol mixture (50:50, *v/v*) for 5 days, and then extraction was performed with an aqueous-ethanol mixture (70:30, *v/v*) in a water bath at 60°C . To characterize the aglycone profile, extracts were hydrolyzed with 2N HCl for 1 h in a water bath.

The leaves of in situ plants were treated similarly at the same time. Each sample presented leaves from 10 plants from a native population grown in a grassland (Novosibirsk, Russia; $54^\circ50'35.4'' \text{ N } 83^\circ06'11.6'' \text{ E}$). Five leafy shoots collected from the middle part of the stem of each plant were combined in the sample.

2.5. Spectrophotometric Analyses

For quantification of photosynthetic pigments, fresh microshoots (0.01 g) were homogenized with 1.5 mL of 95% ethanol and centrifuged at 8000 rpm for 10 min. The supernatant was made up to a volume of 3 mL with 95% ethanol. The absorbance (A) of the extracts was measured at wavelengths of 470, 664, and 648 using a UNICO 2100UV

spectrophotometer (UNICO, Dayton, NJ, USA). Some 95% ethanol was used as a control. The pigment concentration in the plant extract (C , $\text{mg}\cdot\text{L}^{-1}$) was calculated according to H.K. Lichtenthaler [55]. The concentrations of pigments (CP) in tissues were calculated and are expressed in milligrams per gram of FW ($\text{mg}\cdot\text{g}^{-1}$). Samples were analyzed in triplicate.

The total concentration of phenolic compounds was assessed by the Folin–Ciocalteu method [56]. An extract (0.25 mL) was placed in a 10 mL tube; then, 1.25 mL of the Folin–Ciocalteu reagent (diluted 1:9 with distilled water, v/v) was added and the solution was mixed. After 3 min, 1.0 mL of a 7.5% aqueous solution of sodium carbonate was added, and the mixture was incubated at room temperature for 2 h. The absorbance of each sample was measured at 765 nm using a UNICO 2100 UV spectrophotometer. A standard curve was constructed with gallic acid from Sigma-Aldrich (Saint Louis, MO, USA). The results are expressed as mg gallic acid equivalent per gram of DW. Samples were analyzed in triplicate.

The total flavonoid content was measured by the AlCl_3 colorimetric assay according to J. Zhishen et al. [57]. An extract (0.1 mL) was placed into a 10 mL test tube containing 4 mL of distilled H_2O . Then, 0.3 mL of 5% NaNO_2 solution was dispensed into the tube. After 5 min, 0.3 mL of 10% AlCl_3 solution was added to the tube, and the mixture was maintained for 6 min, followed by the addition of 2 mL of 1 M NaOH solution. The total volume was made up to 10 mL with distilled H_2O . The solution was mixed and the absorbance was measured at 510 nm. The total flavonoid content was calculated using a calibration curve, and is expressed as mg quercetin equivalent per gram of DW [58]. Samples were analyzed in triplicate.

2.6. HPLC Analysis

HPLC analysis of the extracts was performed on an Agilent 1200 instrument with a diode array detector (Agilent Technologies, Santa Clara, CA, USA) and ChemStation software (Version Rev. B.04.01. SP1 [647]) for data processing. The chromatographic separation was performed at 25 °C on a Diaspher 110-C18 column (4.6×150 mm, 5 μm internal diameter) (AO BioChemMack ST, Moscow, Russia) [48]. The quantification of compounds was conducted by the external standard method. To prepare calibration curves, standard samples of *p*-coumaric acid, kaempferol, rutin, and formononetin from Sigma-Aldrich (Saint Louis, MO, USA) were used. Standard stock solutions at a concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$ in methanol were employed to obtain calibration curves in the concentration range of 10–100 $\mu\text{g}\cdot\text{mL}^{-1}$.

Identification of the class of unidentified compounds was conducted according to maxima of the UV spectrum. Quantification of unidentified hydroxycinnamic acids and acylated flavonol glycosides was conducted based on the calibration curve of *p*-coumaric acid; flavonoid glycoside concentrations were calculated according to the rutin calibration curve.

Concentrations of the compounds in the extracts are expressed in milligrams per gram of absolute DW of the microshoots. Dry weight concentrations in the samples were calculated by the gravimetric method. Samples were analyzed in triplicate.

2.7. Total Antioxidant Activity

The total antioxidant activity was evaluated in ethanol extracts based on the reduction of Mo(VI) to Mo(V) and subsequent formation of a green phosphate/Mo (V) complex at acidic pH [59]. The extract (0.3 mL) was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The mixture was incubated at 95 °C for 90 min, and the absorbance was measured at 695 nm against a blank (ethanol) after cooling to room temperature. A calibration curve was constructed with ascorbic acid in the concentration range of 2–10 $\mu\text{g}\cdot\text{mL}^{-1}$, and the results are expressed as mg ascorbic acid equivalents (AAE) per gram of DW [60]. Samples were analyzed in triplicate.

2.8. Statistical Analysis

The data were analyzed using STATISTICA 10.0 software (Statsoft Inc., Tulsa, OK, USA). Quantitative data are presented as the mean $\pm$ standard error (SE), and the significance of the differences between data sets was determined by a one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests. Two-way ANOVA was used to analyze the effects of 'TDZ concentration' and 'medium type' on the proliferation of *A. glycyphyllos* axillary microshoots and on their growth and development characteristics. Differences between means were considered statistically significant at $p \leq 0.05$.

3. Results and Discussion

3.1. Seed Germination of *A. glycyphyllos* Depending on Scarification Method and Sterilizing Agent

Although *Astragalus* (Fabaceae) is considered the largest genus of flowering plants in the world [61], an increasing number of *Astragalus* species are regarded as vulnerable, imperiled, or critically imperiled species. This depletion is caused largely by elevated demand and intensive harvesting of these traditional medicinal herbs with high biological value [62]. We found no available information on the cultivation of *Astragalus* plants ex situ, most likely due to hard seed dormancy, which is the main factor hindering the distribution of *Astragalus* plants in nature and in the field [62]. In vitro propagation is one way to increase seed germination and to maintain the natural biodiversity of *Astragalus* species.

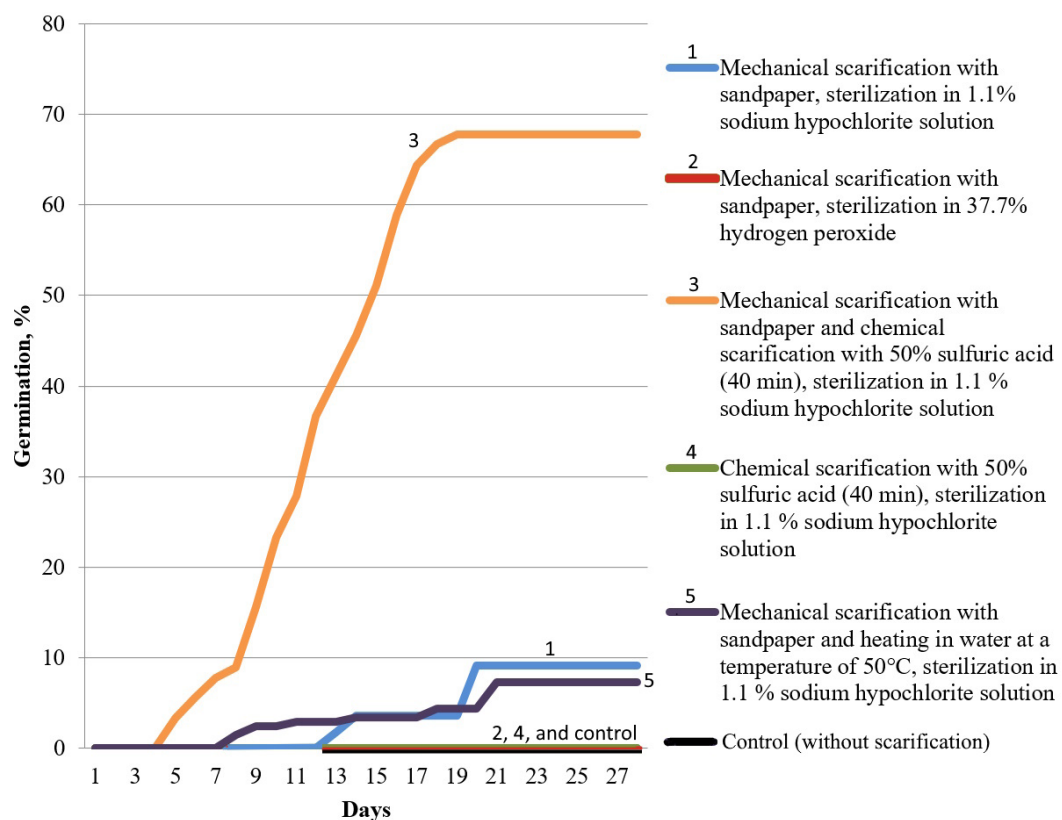
Seeds of representatives of the Fabaceae, possessing a hard, water-impermeable seed coat, are typically characterized by physical dormancy [63,64]. The most effective method to facilitate germination of *A. glycyphyllos* seeds was shown to be the combined application of mechanical and chemical scarification, which achieved the highest final germination percentage (GP) of 67.8%. This high efficacy suggests the necessity of a complex approach to overcome not only the physical dormancy imposed by the dense seed coat but also a pronounced physiological dormancy of the embryo. Physiological dormancy, as described by J.M. Baskin and C.C. Baskin [65], often necessitates combined treatments to effectively activate metabolic processes and stimulate embryo growth. This dual dormancy explains the requirement for aggressive scarification methods, including combining mechanical and chemical actions, to overcome both physical and physiological barriers. Seeds of *A. glycyphyllos* without scarification treatments did not germinate in our experiment.

Surface sterilization was also critical for maintaining seed viability and preventing contamination. Sterilization using a 1.1% sodium hypochlorite solution proved most effective, achieving a germination rate of 16.5% even without prior scarification (Table 1). Variations in the efficacy of the seed sterilization methods may arise from the species-specific structural features and permeability of the seed coat to sodium hypochlorite's active components, as well as the unique microflora associated with *A. glycyphyllos* seeds. These factors collectively determine the species-specific response to the sterilizing agent.

The mean germination time for seeds treated with combined scarification and sterilization with a 1.1% sodium hypochlorite solution was 18.8 days. Following a 4-day period with no germination, radicle emergence was initiated on day 5 and peaked on day 19 (Table 1, Figure 1).

Table 1. Effects of scarification method and sterilizing agent on the germination performance of *A. glycyphyllos*.

Scarification Method	Sterilizing Agent			
	1.1% Sodium Hypochlorite Solution		37.7% Hydrogen Peroxide	
	% of Total Seed Germination (GP)	Mean Seed Germination Time (Mdays)	% of Total Seed Germination (GP)	Mean Seed Germination Time (Mdays)
Control (without scarification)	0	0	0	0
Mechanical with sandpaper	4.9 ± 2.43	8.4 ± 8.35	0	0
Chemical with 50% sulfuric acid (40 min)	0	0	0	0
Mechanical with sandpaper and chemical with 50% sulfuric acid (40 min)	67.8 ± 0.0	16.2 ± 0.47	0	0
Mechanical scarification with sandpaper and heating in water at a temperature of 50 °C	7.3 ± 0.0	15.7 ± 0.24	0	0

**Figure 1.** Seed germination of *A. glycyphyllos* in response to different scarification and sterilization treatments.

Seedling development after germination was significantly influenced by the chosen pretreatment and sterilization methods (Table 2). The most rapid development through the initial stages—primary root emergence, cotyledon growth and development, and true leaf formation—was observed with the combined mechanical–chemical scarification, followed by surface sterilization with a 1.1% sodium hypochlorite solution. With this combined treatment, primary root emergence occurred within 5–8 days, cotyledon growth and development by 10–15 days, and true leaf formation by 17–20 days. In contrast, mechanical scarification followed by surface sterilization with 1.1% sodium hypochlorite solution significantly delayed these stages, with primary root emergence requiring 19–20 days, and true leaf formation taking 28 days. Notably, chemical scarification alone or mechanical scarification combined with sterilization with 37.7% hydrogen

peroxide did not result in germination and, consequently, any subsequent seedling development. Although H_2O_2 has been documented to stimulate seed germination in different species [66], its application in this study did not have a positive effect on *A. glycyphyllos* seeds. High concentrations of H_2O_2 , which might be necessary to overcome the physical seed coat barrier, can induce oxidative stress and damage the embryo before its intrinsic antioxidant system is fully activated [67]. These results highlight the importance of optimizing both seed dormancy breaking and subsequent developmental conditions for effective in vitro propagation of *A. glycyphyllos*.

Table 2. Stages of growth and development of *A. glycyphyllos* plantlets in response to different scarification and sterilization treatments.

Scarification and Sterilizing Treatments		Mechanical Scarification with Sandpaper; Sterilization in 1.1% Sodium Hypochlorite Solution	Mechanical Scarification with Sandpaper; Sterilization in 37.7% Hydrogen Peroxide	Mechanical Scarification with Sandpaper and Chemical Scarification with 50% Sulfuric Acid (40 min); Sterilization in 1.1% Solution of Sodium Hypochlorite	Chemical with 50% Sulfuric Acid (40 min) Scarifications; Sterilization in 1.1% Solution of Sodium Hypochlorite	Mechanical Scarification with Sandpaper and Heating in Water at a Temperature of 50 °C; Sterilization in 1.1% Solution of Sodium Hypochlorite
Plantlet development stages, days	Primary root emergence	19–20	0	5–8	0	8–13
	Cotyledon growth and development	23–27	0	10–15	0	15–20
	True leaf formation	28	0	17–20	0	22–26

3.2. Effects of PGRs and Nutrient Medium Composition on Axillary Microshoot Growth and Development, and Photosynthetic Pigment Content in *A. glycyphyllos* Microshoots

To study the in vitro morphogenic potential of *A. glycyphyllos* explants, various combinations and concentrations of PGRs were tested on different nutrient media. Initially, the explants were cultured on media supplemented with 6-BAP, IAA, 2-iP, and TDZ. However, PGR combinations involving 6-BAP, IAA, and 2-iP did not yield satisfactory results, as evidenced by chlorosis, reduced shoot size, and a low regeneration frequency (Table S1). Due to the lack of positive morphogenetic effects, these PGRs were excluded from further analysis.

Subsequent experiments focused solely on TDZ, a cytokinin whose effectiveness in inducing regeneration in *Astragalus* species, especially those recalcitrant to propagation, has been confirmed in previous studies [68–70]. These studies have reported shoot formation both via callus induction and direct organogenesis. In our experiment, TDZ stimulated the development of axillary microshoots via direct organogenesis (Figure 2).

It was found that the maximum regeneration rate (95%) was achieved on MS medium supplemented with $0.5 \text{ mg} \cdot \text{L}^{-1}$ TDZ (Table 3). However, this high frequency was associated with reduced microshoot quality on standard MS medium. On MS with $2 \times$ Fe-chelate, the highest regeneration frequency reached 85% at the same TDZ concentration, but it was not significantly different from the rate observed at lower TDZ concentrations (0.05 and $0.1 \text{ mg} \cdot \text{L}^{-1}$ TDZ).

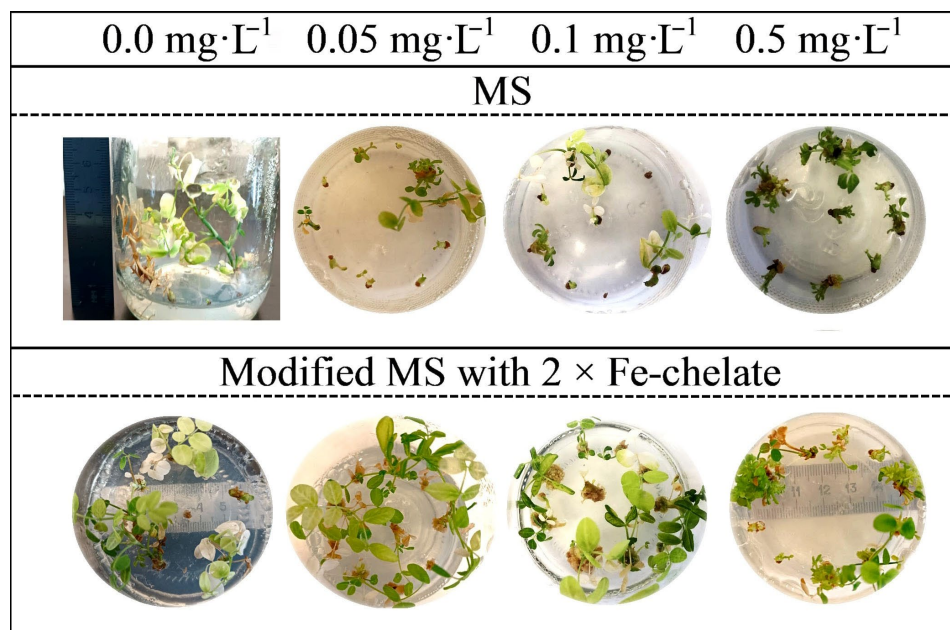


Figure 2. Effect of thidiazuron (TDZ) concentration and medium composition on the quality of *A. glycyphyllos* axillary microshoots on Murashige and Skoog medium (MS) and modified Murashige and Skoog medium with a twofold content of iron chelate (MS with 2× Fe-chelate) supplemented with 0.05–0.5 mg·L^{−1} TDZ after 8-wk cultivation.

Table 3. Effects of TDZ concentration and medium type on proliferation, growth, and development of *A. glycyphyllos* axillary microshoots.

Type of Medium	TDZ Concentration, mg·L ^{−1}	% of Axillary Microshoot Proliferation	Weight of Microshoots, g		Number of Microshoots per Explant	Number of Internodes per Microshoot	Microshoot Length, cm
			Fresh	Dry			
MS	0.0	55.0 ± 0.50 b	0.25 ± 0.05 c	0.08 ± 0.04 bc	0.75 ± 0.19 ab	1.65 ± 0.60 a	1.04 ± 0.40 ab
	0.05	70.0 ± 0.00 ab	0.11 ± 0.02 abd	0.06 ± 0.02 bc	0.70 ± 0.10 ab	0.55 ± 0.22 cd	0.79 ± 0.18 b
	0.1	60.0 ± 0.00 b	0.08 ± 0.01 b	0.04 ± 0.01 bc	0.60 ± 0.10 a	0.25 ± 0.10 d	0.63 ± 0.16 b
	0.5	95.0 ± 0.50 a	0.06 ± 0.03 d	0.04 ± 0.02 c	1.05 ± 0.11 b	0.05 ± 0.05 d	0.68 ± 0.08 b
MS with 2 × Fe-chelate	0.0	55.0 ± 0.50 b	0.05 ± 0.02 d	0.03 ± 0.01 c	0.75 ± 0.18 ab	1.55 ± 0.40 ab	0.64 ± 0.16 b
	0.05	70.0 ± 1.00 ab	0.28 ± 0.06 c	0.20 ± 0.06 a	0.70 ± 0.11 ab	1.20 ± 0.26 abc	1.59 ± 0.29 a
	0.1	80.0 ± 0.00 ab	0.21 ± 0.04 a	0.15 ± 0.03 ab	0.85 ± 0.11 ab	0.75 ± 0.19 bcd	1.68 ± 0.27 a
	0.5	85.0 ± 1.50 ab	0.19 ± 0.06 abc	0.13 ± 0.05 abc	1.05 ± 0.14 b	0.50 ± 0.14 cd	1.15 ± 0.21 ab
Significance of two-way ANOVA							
TDZ concentration		*	ns	ns	*	*	ns
Medium type		ns	*	ns	ns	ns	*
TDZ concentration × Medium type		ns	*	*	ns	ns	*

TDZ—thidiazuron; MS—Murashige and Skoog medium; MS with 2× Fe-chelate—modified Murashige and Skoog medium with a twofold concentration of iron chelate. Data are presented as mean ± SE. Means followed by the same letter do not differ significantly from each other according to Duncan's multiple range test at $p \leq 0.05$. The interaction between TDZ concentration and medium type was analyzed using two-way ANOVA. * Significant at $p \leq 0.05$; ns—not significant.

Although regeneration frequency was high, the FW of microshoots generally decreased with increasing TDZ concentration on both medium types. On standard MS medium, FW decreased from 0.11 g at 0.05 mg·L^{−1} TDZ to 0.06 g at 0.5 mg·L^{−1} TDZ, corresponding to a 45.5% decrease. On MS medium supplemented with 2× Fe-chelate, FW decreased from a peak of 0.28 g at 0.05 mg·L^{−1} TDZ to 0.19 g at 0.5 mg·L^{−1} TDZ, representing a 32.1% decrease.

The highest FW (0.28 g) was observed on the modified medium with $0.05 \text{ mg}\cdot\text{L}^{-1}$ TDZ, which was 460% higher than the control on the same medium, indicating the production of healthier and larger microshoots under these conditions. Dry weight followed a similar trend, with the highest value (0.20 g) also obtained on MS with $2\times$ Fe-chelate and $0.05 \text{ mg}\cdot\text{L}^{-1}$ TDZ—a 566.67% increase over the corresponding control.

Furthermore, lower TDZ concentrations generally promoted better shoot elongation, as evidenced by a higher number of internodes. On the modified MS medium, microshoot length initially increased with TDZ concentration up to $0.1 \text{ mg}\cdot\text{L}^{-1}$ (1.68 cm), and then slightly decreased at $0.5 \text{ mg}\cdot\text{L}^{-1}$ (1.15 cm). This observation suggests that the twofold Fe-chelate concentration may mitigate the typical inhibitory effect of TDZ on microshoot elongation at moderate concentrations (Table 3). These findings indicate that optimal microshoot quality and biomass accumulation were achieved not by high TDZ on standard MS, but rather by the use of modified MS combined with moderate TDZ concentrations ($0.05\text{--}0.1 \text{ mg}\cdot\text{L}^{-1}$).

Possibly, this tolerance is attributed to the specific expression patterns of cytokinin signaling genes in this species [71]. Furthermore, the physiological properties of *A. glycyphyllos*, such as potentially elevated levels of endogenous cytokinins, may reduce its sensitivity to exogenous growth regulators. Additionally, features of iron metabolism likely influence cellular differentiation and in vitro organogenesis.

A two-way ANOVA revealed that TDZ concentration had a significant effect on the regeneration frequency, the number of microshoots per explant, and the number of internodes per microshoot ($p \leq 0.05$). In contrast, medium type significantly influenced only the microshoot FW and length ($p \leq 0.05$). No significant effect of medium type was observed on regeneration frequency, DW, the number of microshoots per explant, or internode number. Importantly, a significant interaction between TDZ concentration and medium type was found for microshoot FW, DW, and length ($p \leq 0.05$). This indicates that the effect of TDZ concentration on these parameters is not independent but varies significantly depending on the type of nutrient medium used. In contrast, no significant interaction was observed for regeneration frequency, the number of microshoots per explant, or the number of internodes per microshoot.

The highest concentrations of photosynthetic pigments were associated with high TDZ concentrations ($0.5 \text{ mg}\cdot\text{L}^{-1}$) and an increased iron concentration (Figure 3). Iron is a crucial element in the physiology of *A. glycyphyllos*, participating in oxidation–reduction processes during chlorophyll synthesis. The key enzymes involved in chlorophyll formation contain iron atoms in their active site. Of particular importance is the cytochrome system, where iron–porphyrin complexes ensure electron transfer through changes in the iron’s oxidation state [72,73].

The chlorophyll a/b ratio on standard MS medium (4.6) was significantly higher than on the modified MS (1.9), indicating basic physiological differences in the pigment complex even without the addition of TDZ. On standard MS medium, TDZ caused a 22–55% decrease in total chlorophyll content relative to the control (MS without TDZ), reaching a minimum at $0.1 \text{ mg}\cdot\text{L}^{-1}$. In contrast, on the modified MS medium, TDZ stimulated an increase in total chlorophyll by 5–66% compared to the control on the same medium. The maximum concentrations of total chlorophyll a and b ($0.67 \text{ mg}\cdot\text{g}^{-1}$) and carotenoids ($0.18 \text{ mg}\cdot\text{g}^{-1}$) were achieved on the MS with $2\times$ Fe-chelate supplemented with $0.5 \text{ mg}\cdot\text{L}^{-1}$ TDZ. The presence of iron chelate in the nutrient medium not only mitigates the inhibitory effect of TDZ on shoot elongation (as demonstrated previously) but also eliminates its negative impact on pigment composition.

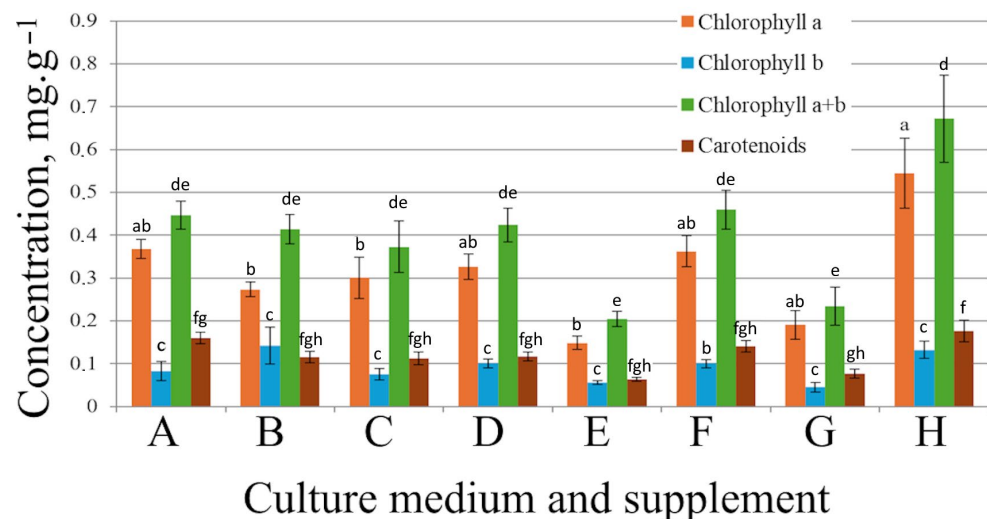


Figure 3. Concentrations of photosynthetic pigments in *A. glycyphyllos* microshoots depending on thidiazuron (TDZ) and iron chelate concentration in the medium. Data are presented as mean $\pm$ SE. Means followed by the same letter do not differ significantly from each other according to Duncan's multiple range test at $p \leq 0.05$. Abbreviations: A—MS, 0.0 mg·L⁻¹ TDZ; B—MS with 2× Fe-chelate, 0.0 mg·L⁻¹ TDZ; C—MS, 0.05 mg·L⁻¹ TDZ; D—MS with 2× Fe-chelate, 0.05 mg·L⁻¹ TDZ; E—MS, 0.1 mg·L⁻¹ TDZ; F—MS with 2× Fe-chelate, 0.1 mg·L⁻¹ TDZ; G—MS, 0.5 mg·L⁻¹ TDZ; H—MS with 2× Fe-chelate, 0.5 mg·L⁻¹ TDZ; MS—Murashige and Skoog medium; MS with 2× Fe-chelate—modified Murashige and Skoog medium with a twofold concentration of iron chelate.

The results are consistent with the well-established role of TDZ as a potent cytokinin capable of inducing shoot regeneration in numerous plant species. Literature data confirm that TDZ effectively induces shoot formation in the range of 1 nM to 10 μ M [74], with the maximum number of regenerants often achieved at concentrations of 0.1 to 1.0 μ M [51]. Importantly, TDZ exhibits a dual role in morphogenesis: lower concentrations promote axillary shoot proliferation, while higher concentrations induce adventitious shoot formation. Our applied concentration of 0.05 mg·L⁻¹ TDZ (approximately 0.23 μ M) falls within this optimal range, confirming its efficacy for inducing microshoot development in *A. glycyphyllos*. One of the key aspects of our study is the effect of TDZ on shoot elongation. It is widely reported that while inducing regeneration, high concentrations of TDZ often inhibit the subsequent growth and development of regenerants [75], leading to shortened microshoots, a reduced internode number, and abnormalities such as hyperhydricity and fasciation [76]. Our observations on standard MS medium confirmed this trend, as shoot length generally decreased with increasing TDZ concentration. This is consistent with the findings of B.N.S. Murthy et al. [77], who reported growth retardation and other adverse effects of TDZ. However, our study revealed a significant synergistic effect of the modified MS medium with twofold the concentration of iron chelate. On this modified medium, microshoot length peaked at 1.68 cm at 0.1 mg·L⁻¹ TDZ, which was significantly longer than that on standard MS. This result demonstrates that an elevated iron chelate concentration can mitigate the typical inhibitory effect of TDZ on shoot elongation, thereby promoting more vigorous growth at moderate TDZ levels. This effect may be attributed to the stress response induced by TDZ, which is characterized by the accumulation of mineral ions and stress-related metabolites [77,78]. Supplemental iron may help plants cope with this stress by improving metabolism or supporting key physiological processes required for elongation. TDZ may also negatively impact endogenous auxin and cytokinin levels [79], and improved iron nutrition may partially offset this imbalance. Thus, although high TDZ concentrations on standard MS can maximize explant regeneration rates, our protocol on modified MS medium with a twofold concentration of iron chelate and a moderate

TDZ concentration ($0.05\text{--}0.1\text{ mg}\cdot\text{L}^{-1}$) proved optimal for obtaining high-quality, elongated microshoots with improved biomass. This provides a valuable strategy for overcoming the limitations of TDZ associated with subsequent growth and plantlet quality, which is a common problem in micropropagation. Our study demonstrates that not only TDZ concentration but also modifications to the basal medium play a crucial role in achieving the desired morphogenetic response and the quality of the resulting plants.

3.3. Concentrations of Phenolics and Flavonoids and Their Associated Antioxidant Activity in *A. glycyphyllos* Microshoots

The total phenolic concentrations in the aboveground part of *A. glycyphyllos* microshoots were comparable to or higher than those in plants under in situ conditions (Figure 4). The highest level of total phenolic concentration ($5.96\text{ mg}\cdot\text{g}^{-1}$) was achieved on the modified MS medium supplemented with a relatively low TDZ concentration ($0.05\text{ mg}\cdot\text{L}^{-1}$). A similarly high concentration was registered on a medium without TDZ supplemented with a twofold concentration of iron chelate ($5.89\text{ mg}\cdot\text{g}^{-1}$). This may indicate an increased sensitivity of this species to cytokinins and the important role of iron in stabilizing chloroplast membranes, where some of the reactions of phenolic compound biosynthesis occur [71].

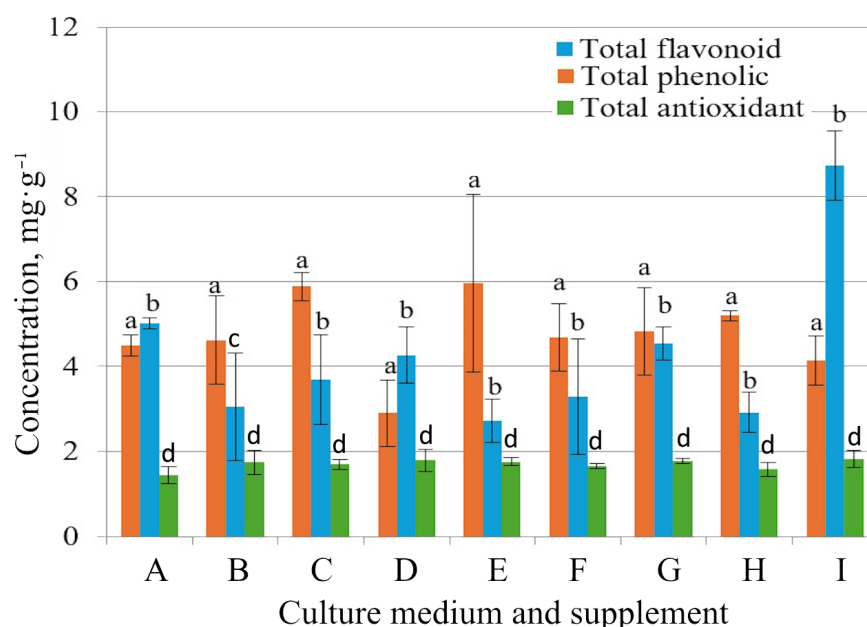


Figure 4. Total phenolic (gallic acid equivalent) and total flavonoid (quercetin equivalent) concentrations, and total antioxidant activity (ascorbic acid equivalent) of microshoots and in situ plants of *A. glycyphyllos*, depending on concentrations of thidiazuron (TDZ) and iron chelate in the medium. Data are presented as mean $\pm$ SE. Means followed by the same letter do not differ significantly from each other according to Duncan's multiple range test at $p \leq 0.05$. Abbreviations: A—in situ plants; B—MS, $0.0\text{ mg}\cdot\text{L}^{-1}$ TDZ; C—MS with $2\times$ Fe-chelate, $0.0\text{ mg}\cdot\text{L}^{-1}$ TDZ; D—MS, $0.05\text{ mg}\cdot\text{L}^{-1}$ TDZ; E—MS with $2\times$ Fe-chelate, $0.05\text{ mg}\cdot\text{L}^{-1}$ TDZ; F—MS, $0.1\text{ mg}\cdot\text{L}^{-1}$ TDZ; G—MS with $2\times$ Fe-chelate, $0.1\text{ mg}\cdot\text{L}^{-1}$ TDZ; H—MS, $0.5\text{ mg}\cdot\text{L}^{-1}$ TDZ; I—MS with $2\times$ Fe-chelate, $0.5\text{ mg}\cdot\text{L}^{-1}$ TDZ; MS—Murashige and Skoog medium; MS with $2\times$ Fe-chelate—modified Murashige and Skoog medium with a twofold concentration of iron chelate.

The total flavonoid concentration in the microshoots either corresponded to or was lower than that in the leaves of in situ plants, with the exception of microshoots grown on MS medium with a twofold concentration of iron chelate and the highest TDZ concentration ($0.5\text{ mg}\cdot\text{g}^{-1}$). Microshoots grown on this medium showed the maximum flavonoid concentration ($8.74\text{ mg}\cdot\text{g}^{-1}$) (Figure 4).

Total flavonoid concentrations in aboveground parts of *Astragalus* plants, which have been determined previously, varied significantly. Leaves of *A. glycyphyllos* plants grown in Poland exhibited 2.43 and 1117.62 μg of quercetin equivalents per gram with aqueous methanol and water extraction, respectively [19]. Meanwhile, under Novosibirsk conditions, 0.88–1.95 mg of rutin equivalent per gram were revealed in the aboveground part of *A. glycyphyllos* [80]. The presented result for native plants ($5.01 \text{ mg}\cdot\text{g}^{-1}$) is higher than these values. In addition, the highest total flavonoid content, which was registered in the microshoots grown on a medium supplemented with $0.5 \text{ mg}\cdot\text{L}^{-1}$ TDZ ($8.74 \text{ mg}\cdot\text{g}^{-1}$), may indicate a stressful effect of a high TDZ concentration.

The level of total antioxidant activity of the in vitro microshoots grown with a majority of the supplements ($1.66\text{--}1.83 \text{ mg AAE}\cdot\text{g}^{-1}$) significantly surpassed that of plants grown under in situ conditions ($1.44 \text{ mg AAE}\cdot\text{g}^{-1}$). The highest antioxidant activity ($1.83 \text{ mg AAE}\cdot\text{g}^{-1}$), correlating with the highest flavonoid level, was recorded in the microshoots cultivated on a medium supplemented with $0.5 \text{ mg}\cdot\text{L}^{-1}$ TDZ.

The phenolic profile of *A. glycyphyllos* microshoots cultivated on the medium that exhibited the highest increase in microshoot biomass was examined (MS with $2\times$ Fe-chelate, $0.05 \text{ mg}\cdot\text{L}^{-1}$ TDZ) (Table 3). Hydroxycinnamic acids, kaempferol glycosides, and acylated flavonoids were primarily detected in aqueous ethanol extracts of the aboveground part of the microshoots (Figure 5, Table 4). The contents of hydroxycinnamic acids, flavonoid glycosides, and acylated flavonoids in the extract were 1.72 , 1.51 , and $2.76 \text{ mg}\cdot\text{g}^{-1}$ (Table 4). Accordingly, acylated flavonoids showed the highest abundance in microshoot tissues. The total phenolic content in microshoots was $5.99 \text{ mg}\cdot\text{g}^{-1}$. Kaempferol was the main component in the hydrolyzed extract ($0.98 \text{ mg}\cdot\text{g}^{-1}$). Meanwhile, formononetin in a modest amount ($0.09 \text{ mg}\cdot\text{g}^{-1}$) was detected (Figure S1).

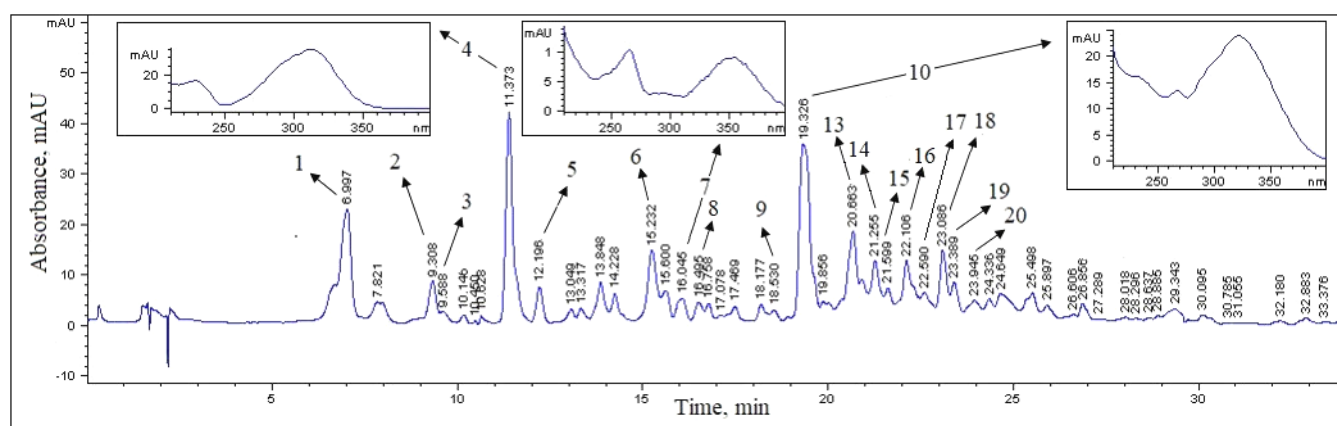


Figure 5. Representative section of an HPLC chromatogram of an aqueous ethanol extract of *A. glycyphyllos* microshoots (detection at 325 nm). The number of peaks is presented in Table 4.

The major compounds of the microshoots and in situ *A. glycyphyllos* plants generally coincided. Their shared main components included hydroxycinnamic acid 1, *p*-coumaric acid, and acylated flavonol glycosides 6, 8, and 13. The in situ plants, however, contained rutin and flavonol glycoside 12, which were not detected in the microshoots. On the other hand, the microshoots contained flavonoids 3 and 7, which were not found in in situ plants. Moreover, the concentrations of the majority of phenolic compounds in the microshoots were higher compared to those in in situ plants analyzed simultaneously (Table 4). This phenolic profile shows similarity to that of *A. glycyphyllos* plants from a considerably distant region—western Ukraine [34]. It was also characterized by a number of hydroxycinnamic acids, with *p*-coumaric acid as the main component. The concentration of *p*-coumaric

acid recorded for plants from Ukraine ($1.3 \text{ mg}\cdot\text{g}^{-1}$) was higher than that in our microshoots and in situ plants.

Table 4. Concentrations of phenolic compounds in native and hydrolyzed extracts of microshoots and leaves of in situ plants of *A. glycyphyllos* ($\text{mg}\cdot\text{g}^{-1}$ dry weight).

No	Compound	t_R , Minutes	$\lambda_{\max}$, nm	Microshoots	In Situ Plants
Extract					
Hydroxycinnamic acids					
1	Hydroxycinnamic acid 1	6.9	220, 295sh., 314	0.46 ± 0.03	0.43 ± 0.02
2	Hydroxycinnamic acid 2	9.3	200, 309	0.12 ± 0.01	0.08 ± 0.00
4	<i>p</i> -Coumaric acid	11.4	229, 313	0.56 ± 0.02	0.32 ± 0.01
5	Hydroxycinnamic acid 5	12.2	219, 298sh., 325	0.08 ± 0.01	0.08 ± 0.00
19	Hydroxycinnamic acid 19	23.4	238, 295sh., 329	0.11 ± 0.01	0.06 ± 0.00
	Total hydroxycinnamic acids *			1.72 ± 0.14	1.71 ± 0.12
Flavonoid glycosides					
3	Flavone glycoside 3	9.6	265, 350	0.25 ± 0.02	ND
7	Flavonol glycoside 7	16.0	265, 295sh., 355	0.40 ± 0.03	ND
8	Flavonol glycoside 8	16.5	268, 345	0.26 ± 0.01	0.24 ± 0.01
9	Kaempferol glycoside	18.5	266, 350	0.14 ± 0.01	0.14 ± 0.01
11	Rutin	18.9	256, 357	ND	0.04 ± 0.00
12	Quercetin glycoside 12	20.4	252, 350	ND	0.36 ± 0.02
20	Kaempferol glycoside 20	23.9	268, 348	0.46 ± 0.02	0.01 ± 0.00
	Total flavonoid glycosides			1.51 ± 0.09	0.79 ± 0.03
Acylated kaempferol glycosides					
6	Acylated kaempferol glycoside 6	15.2	218, 268, 318	0.25 ± 0.02	0.19 ± 0.01
10	Acylated kaempferol glycoside 10	19.3	232sh., 269, 321	0.73 ± 0.05	0.11 ± 0.00
13	Acylated kaempferol glycoside 13	20.7	231, 270, 318	0.30 ± 0.02	0.23 ± 0.01
14	Acylated kaempferol glycoside 14	21.3	231, 271, 319	0.21 ± 0.01	0.06 ± 0.00
15	Acylated kaempferol glycoside 15	21.6	266, 320	0.09 ± 0.01	0.08 ± 0.00
16	Acylated kaempferol glycoside 16	22.1	231, 270, 319	0.27 ± 0.02	0.08 ± 0.00
17	Acylated kaempferol glycoside 17	22.6	242, 265, 321	0.10 ± 0.01	0.03 ± 0.00
18	Acylated kaempferol glycoside 18	23.0	225, 269, 315	0.21 ± 0.02	0.06 ± 0.00
	Total acylated kaempferol glycosides *			2.76 ± 0.18	1.12 ± 0.05
	Total phenolic compounds			5.99 ± 0.30	3.62 ± 0.15
Hydrolyzed extract					
	Kaempferol	27.7	268, 368	0.98 ± 0.02	0.22 ± 0.01
	Formononetin	29.5	250, 302 sh.	0.09 ± 0.00	ND

Data are presented as mean $\pm$ SE. * Total of all detected components calculated as *p*-coumaric acid equivalents. Abbreviations: t_R —retention time; $\lambda_{\max}$ —absorption maxima; ND—not detected.

Another phenolic profile with a lack of *p*-coumaric acid and a significant level of rutin was revealed by S. Shahrivari-Baviloliaei et al. [19]. The concentration of total phenolic acids, determined by spectrophotometry and HPLC analysis, was comparable to that of native plants from our study. A similar phenolic profile was demonstrated by P. Popova et al. for *A. glycyphyllos* microshoots [38]. The highest concentration of flavonoids ($2.37 \text{ mg}\cdot\text{g}^{-1}$ DW), which was significantly lower than that in the microshoots in our work ($4.27 \text{ mg}\cdot\text{g}^{-1}$ DW), was observed by the authors in callus cultures cultivated on $\text{G48} + 2\text{Ca}^{2+}$ medium [38]. We did not find a significant amount of quercetin glycosides in the microshoots, but they were detected in the in situ plants. However, their concentration was moderate ($0.4 \text{ mg}\cdot\text{g}^{-1}$).

On the other hand, our culture produced a number of acylated kaempferol glycosides ($2.76 \text{ mg}\cdot\text{g}^{-1}$). They were also revealed in the native plants at lower concentrations. Acylated kaempferol glycosides of various structures have been identified in representatives of the genus *Astragalus* [81,82]. These compounds, combining organic or phenolic acids and flavonoids in their molecules, require accurate identification and evaluation of their biological properties. Acylated compounds have been found to possess higher stability and bioactivity [83]. The antimicrobial, anti-parasitic, anti-inflammatory, anti-nociceptive,

analgesic, and anti-complementary properties of acylated flavonoids have been comprehensively reviewed [84]. The difference in phenolic composition of the microshoot cultures may be related to the variation of medium supplements, other culture conditions, and geographic origin (according to literature data) of *A. glycyphyllos* plant material.

The concentration of formononetin in the microshoots from our study was comparable to that reported by B. Butkute et al. [23]. The authors observed a significant variation in isoflavone levels in plant tissues throughout the vegetation period. The total biochanin A, formononetin, and genistein in the aboveground part of field-grown *A. glycyphyllos* plants reached a maximum at the stem elongation stage ($0.204 \text{ mg} \cdot \text{g}^{-1} \text{ DW}$) [23].

Thus, the established in vitro culture, combining a TDZ concentration of $0.05 \text{ mg} \cdot \text{L}^{-1}$ with a twofold increase in iron chelate concentration, induced a high accumulation of flavonoids ($4.27 \text{ mg} \cdot \text{g}^{-1} \text{ DW}$), as well as significant levels of total phenolics ($5.99 \text{ mg} \cdot \text{g}^{-1}$), and antioxidants ($1.83 \text{ mg AAE} \cdot \text{g}^{-1}$) in the microshoot tissues. The highest levels of flavonol glycosides, quercitrin ($8.1 \text{ mg} \cdot \text{g}^{-1}$) and isoquercitrin ($5.3 \text{ mg} \cdot \text{g}^{-1}$), were achieved by I. Ionkova in cell cultures of *A. missouriensis* on medium supplemented with $1.0 \text{ mg} \cdot \text{L}^{-1}$ 1-naphthaleneacetic acid, $2.0 \text{ mg} \cdot \text{L}^{-1}$ kinetin, and 6% (*w/v*) sucrose [43].

In the available literature, we could not find any references on the application of iron chelate in tissue culture of *Astragalus* plants or on its possible influence on the physiological state and accumulation of bioactive compounds. However, its crucial role in plant development has been known [85,86], and iron chelate has been shown to enhance the content of flavonoids and phenolic acids in legume sprouts [87]. We applied this component to boost the physiological state of the plantlets, which had shown reduced chlorophyll content in our preliminary experiments. In the current study, iron chelate exhibited a positive effect on both chlorophyll content and flavonoid content in the tested microshoots. Therefore, iron chelate application may be of interest to scientists concerned with *Astragalus* propagation. Investigating the correlation between iron chelate, physiological state, and phenolic compounds in in vitro culture could be a promising direction for future research.

In addition, the results of the present study reveal a peculiarity of flavonoid accumulation in the *Astragalus* microshoots. The detection of only one flavonol aglycone—kaempferol—in the hydrolyzed extracts and of numerous kaempferol glycosides in the extracts indicates that kaempferol is the main flavonol compound in the established in vitro culture.

In the study by P. Popova et al. (2020), only two chromatographic peaks, corresponding to rutin and camelliaside A, were identified [38]. Concentrations were determined only for these two compounds. Thus, the previous results do not provide insight into the complete chromatographic profile of *A. glycyphyllos*, including the main aglycone components, the ratios between components, and their flavonoid classes. Our findings clearly indicate that kaempferol is the main aglycone component of the microshoots, present in the form of glycosides and acylated glycosides. Further LC-MS or other chemical assays are necessary to unequivocally identify these glycosides, although their chemical class is clear.

4. Conclusions

The established in vitro culture contributes to improving the propagation efficiency of *A. glycyphyllos* plants, which can be considered a source of phenolic compounds, mainly *p*-coumaric acid and kaempferol derivatives. In this culture, iron chelate, along with a low concentration of thidiazuron, stimulated the accumulation of phenolic compounds and antioxidants. Future studies will focus on inducing quercetin biosynthesis in the plantlets through variations in environmental factors (light intensity, temperature, salt concentration) and the application of elicitors. Moreover, a comprehensive study of the dynamics of the main phenolic compounds throughout the entire clonal micropropagation, including ex

vitro and in vivo stages, is necessary to enhance the biological value of in vitro-derived *Astragalus* plants.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijpb17040025/s1>. Figure S1: UV spectra of kaempferol and formononetin in hydrolyzed extracts (A) and kaempferol glycoside and acylated flavonol glycoside (B) in extracts of *A. glycyphyllos* microshoots; Table S1: Effect of concentration of plant growth regulators and type of medium on the proliferation of *A. glycyphyllos* axillary microshoots and the growth and development characteristics of microshoots.

Author Contributions: Conceptualization, E.A. and O.K.; methodology, E.A. and O.K.; validation, E.K. and O.K.; formal analysis, E.A., E.K. and O.K.; investigation, U.P., O.K. and E.A.; resources, E.A.; data curation, E.A., E.K. and O.K.; writing—original draft preparation, U.P., E.A., E.K. and O.K.; writing—review and editing, E.A., E.K. and O.K.; visualization, E.A. and O.K.; supervision, E.A.; project administration, E.A.; funding acquisition, E.A. and O.K. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Raw data of this study are available upon request from the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations were used in this manuscript:

MS	Murashige and Skoog medium
MS with 2× Fe-chelate	modified Murashige and Skoog medium with a twofold concentration of iron chelate
6-BAP	6-benzylaminopurine
IAA	indole-3-acetic acid
2-iP	6-(2-isopentenyl)adenine
TDZ	thidiazuron
HPLC	high-performance liquid chromatography

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