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Establishment and analysis of tissue and fast-growing normal root cultures of four *Gentiana* L. species, rare highland medicinal plants

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Aim. To obtain tissue and isolated root cultures of four *Gentiana* L. species from Ukraine (*G. lutea* L., *G. punctata* L., *G. acaulis* L. and *G. asclepiadea* L.) and study peculiarities of their growth and content of flavonoids and xanthonenes. **Methods.** *In vitro* culture, chromatography, spectrophotometry and statistical methods. **Results.** The conditions were developed for callus induction, proliferation, and long-term maintenance of fast-growing root cultures from gentians. A comparative study of total flavonoid and xanthone content in the cultured tissues, isolated cultured roots and wild plants of gentians was carried out. The capacity was ascertained for synthesizing these biologically active substances *in vitro*. The content of compounds varied both in calli and isolated roots derived from the plants of different gentian species, and in tissue and organ cultures of these species. The morphogenic and non-morphogenic cultures showed much lower flavonoid and xanthone content than the shoots of intact plants, but similar to that of natural roots. The fast-growing normal root cultures displayed higher concentrations of these biologically active compounds than the callus tissues in most cases. **Conclusions.** A high yield of biomass from gentian cultures *in vitro* and their ability to synthesize and accumulate flavonoids and xanthonenes make them as a promising source of these biologically active compounds.

Keywords: *Gentiana* L. species, callus induction and proliferation, fast-growing normal root cultures, flavonoid and xanthone content, source of biologically active compounds.

Abbreviations

CI	— Callus induction	PGR	— Plant growth regulators
GI	— Growth index	Kin	— Kinetin
B₅	— Gamborg and Eveleigh (1968) medium	BAP	— 6-Benzylaminopurine
MS	— Murashige and Skoog (1962) medium	2,4-D	— 2,4-Dichlorophenoxyacetic acid
MS/2	— MS with twice decreased macro- and micro-salts concentrations	NAA	— α -Naphthaleneacetic acid
		BAS	— Biologically active substances

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Introduction

Gentiana L., the largest genus of Gentianaceae Juss. family, includes about 400 species [1, 2]. In Ukraine *Gentiana* genus is represented by 10 species. Most of them are spread throughout the highland regions in the Carpathians [3, 4]. Gentians are extensively used for traditional and folk medicine in the world and, therefore, are currently the subject of intensive phytochemical investigations. Their medicinal activity results from several classes of secondary metabolites produced and stored in various parts of the plants. These are such compounds as alkaloids, iridoids, xanthonenes, flavonoids, carbohydrates, aromatic and nitrogen-bearing compounds, pectic substances, ascorbic acid, catechines *etc.* [5, 6].

Flavonoids and xanthonenes are of special interest as they are characterized by a wide spectrum of influence on humans.

Flavonoids have anti-inflammatory, anti-spasmodic, capillary strengthening, choleretic, diuretic, anti-sclerotic, antineoplastic, anti-oxidant, detoxifying and other effects. They affect the enzyme systems, immune and metabolic processes, thicken membranes, prevent storage of free radicals in tissues, improve the motor activity, secretory and absorbing functions of alimentary canal, *etc.* [5, 6].

The qualitative and quantitative content of xanthonenes is an important chemotaxonomic feature for *Gentianaceae* [6, 7]. Naturally occurring xanthonenes are widely reported for their significant biological and pharmacological properties [6]. Xanthonenes of *Gentiana* act as antioxidants, antidepressants, vasodilators, inhibitors of acetylcholinesterase activities, ex-

hibit antimicrobial and antiviral, cardiogenic and tuberculostatic effects [8–11].

Due to the uncontrolled use of roots for glucoside extraction and for liquor manufacturing, some of the species, like *G. lutea* are close to extinction in their natural habitats. *G. lutea* and related gentians are under protection as endangered species in most European countries [12], which is one more reason for expediency of their introduction and growing *in vitro* culture.

In vitro culture of tissues and organs is one of the alternative sources of medicinal raw material with limited natural store. At the same time, some changes in secondary metabolism take place under the conditions of growing *in vitro*. These include a lack of cell differentiation in callus and suspension cultures, differences of the biosynthetic potential of tissue and organ cultures derived from the various types of explants and plant genotypes, and structural and functional rearrangements of the cell genome in culture [13]. During the subcultivation process or in the initial callus cultures in many cases one can observe a considerable decrease of BAS and their synthesis may regenerate only after formation of some morphogenic structures, i. e. after the restoration of organism regulation level [13]. Therefore, for biosynthesis and accumulation of valuable secondary metabolites, along with the non-morphogenic tissue culture a morphogenic culture, in particular isolated roots, is promising.

Scientific literature describes the isolated roots ability to synthesize the biologically active substances immanent to roots of whole plants [14]. To increase the productivity of isolated root culture, the transformation due to

Agrobacterium rhizogenes is used. Herewith the so called “hairy roots” are obtained that are characterized by a high growth rate in the phytohormones free medium, the genetic stability and capacity of intense synthesis of valuable secondary metabolites [14, 15]. Such method of obtaining isolated root culture was used for gentians [16–19]. The biosynthetic activity was investigated in some gentians hairy root cultures [20–24].

The objective of this work was to obtain tissue and isolated root cultures from some *Gentiana* species and to study peculiarities of their growth and content of flavonoids and xanthonenes in them.

Materials and Methods

Plants of four *Gentiana* species were obtained from seeds harvested in different localities

(Table 1), and grown in aseptic conditions *in vitro*.

For **callus induction** we used root explants of axenic plants of 9 genotypes of 4 *Gentiana* species (Table 1). Every experiment variant included testing 100–150 explants. The basic media used for callus induction and proliferation were B₅ (only for *G. asclepiadea* L.) [25], and MS-based medium [26] with full (MS) and twice decreased macro- and microsalts concentrations (MS/2), supplemented with various combinations and concentrations of plant growth regulators (PGR) including Kin, BAP, (2,4-D and NAA. The cultures were incubated in darkness at 25–26.5°C, with subculture every 4 weeks. The percentage of callus formation was recorded after 3 weeks of cultivation. Callus induction (CI) frequency was determined as follows:

Table 1. Habitat of the investigated gentians

Species	Locality	Altitude (m)	Symbolic notation
<i>G. lutea</i>	Rohneska mountain valley (Chornohora range, Rakhiv district, Transcarpathian region)	1650	G.l.R
	Troyaska mountain (Svydovets range, Rakhiv district, Transcarpathian region)	1695	G.l.Tr
	Pozhyzhevskaya mountain (Chornohora range, Nadvirna district, Ivano-Frankivsk region)	1420	G.l.P
<i>G. punctata</i>	Breskul mountain (Chornohora range, Nadvirna district, Ivano-Frankivsk region)	1790	G.p.Br
	Troyaska mountain (Svydovets range, Rakhiv district, Transcarpathian region)	1704	G.p.Tr
<i>G. acaulis</i>	Turkul mountain (Chornohora range, Rakhiv district, Transcarpathian region)	1750	G.ac.T
	Rebra mountain (Chornohora range, Rakhiv district, Transcarpathian region)	2001	G.ac.Reb
<i>G. asclepiadea</i>	Pozhyzhevskaya mountain (Chornohora range, Nadvirna district, Ivano-Frankivsk region)	1424	G.asc.P
	Velyka Myhla mountain (Gorgany range, Dolyna district, Ivano-Frankivsk region)	950	G.asc.M

$$CI = \frac{Nc}{N} \cdot 100\%, \quad (1)$$

where Nc — number of explants developing callus; N — number of explants cultured.

To determine the optimal conditions for callus proliferation, 0.25–0.35 g portions were separated from explants and placed on nutrient media with various combinations of BAP and 2,4-D (Fig. 1A).

The growth index (GI) according to callus fresh weight was determined after 21 days according to the formula:

$$GI = \frac{M \cdot m}{m}, \quad (2)$$

where M — callus weight after 21 days; m — initial callus weight.

Fast-growing isolated root cultures were obtained from plants of different genotypes of four *Gentiana* species (Table 1).

Root apices each 1.5 cm long and 20–30 mg were used as initial explants for obtaining isolated root cultures, the inocula being taken from two-month axenic plants. Root cultures were obtained in two stages. At the first stage, the inocula were cultured for 2–3 weeks in MS/2 or B₅ (only for *G. asclepiadea*) liquid

nutrient medium, supplemented with various concentrations of NAA and BAP or Kin (Fig. 1B). At the second stage, cultured roots with side rootlets were cultured in MS/2 or B₅ liquid medium without growth regulators for 2–3 weeks (Fig. 1C). The pH of both media was 5.6 ± 0.2 before autoclaving.

With regard to data concerning the calciphilous nature of the species, an increased CaCl₂ concentration was used for *G. acaulis* [27].

The volume of medium at both stages of culture was 50 ml, in 250 ml wide-mouth Erlenmeyer flasks with constant stirring on a shaker with 60–80 vibrations/min without light at 24 ± 2 °C.

The inocula before placing in the medium and isolated root cultures after 4–6 weeks of culture (the first and second culture stages) were weighed in axenic conditions with further determination of their growth index by fresh weight according to Eq. 2, where M — isolated root weight after 4–6 weeks of culture, and m — initial inoculum weight.

Additionally to growth index, the mean number of side rootlets per cultured inocula were determined as well as the mean length after the first and second stages of culture.



Fig. 1. *G. lutea* callus (A) and isolated root culture at I (B) and II (C) stages of cultivation.

These parameters are important to characterize the isolated root cultures, as they indicate the ability of roots to grow continuously [27]. Isolated tips of the main roots are not capable of growing in culture for a prolonged period and their continued use as inocula is accompanied by “ageing” of meristems resulting in death. This excludes the possibility to obtain sufficient biomass. By using the tips of side rootlets as inocula due to their number and vitality, the growth culture cycle is considerably extended.

The material for the research involved tissue cultures of root origin derived from plants of different genotypes at the 11–15th passages of growth (Table 1) and fast-growing root cultures, as well as wild plants of the target species.

Xanthenes and flavonoids were quantified in isolated root cultures after the second stage of growth, in callus tissues at the end of the 11–15th passage, and in wild plants within the fruiting phase. To evaluate the biosynthetic activity of callus tissues, they were compared by the BAS content with shoots and roots of plants from natural localities.

Total xanthone amount was determined by the modified chromatospotometric method [28]. Mangiferin was used as a standard. The air-dried raw material was hydrolyzed in a mixture of acetone and water 1:1 (v:v; mixture A), containing 5 % HCl, on a water bath for 1 hour. Using micropipette with the investigated extract and standard mangiferin solution (0.03 ml of each), on the plate with cellulose of 20 × 20 cm in size we formed two-centimetre stripes three times. One more stripe was left clean and used as a negative control. After chromatography in a saturated

15 % solution of acetic acid, the plates were analyzed under ultraviolet light (360 nm). At the level of standard mangiferin spots of the stripes with the investigated extract there were marked zones, containing xanthenes. The stripe with negative control had an equal in area spot of cellulose used for the preparation of control solution. All the marked areas of cellulose were quantitatively transferred and then desorbed in 10 ml of mixture A and periodically shaken for 15 minutes. The optical density of filtrated solutions was determined spectrophotometrically at 369 nm against a reference solution.

The total xanthone content was calculated from a standard mangiferin curve and expressed as the mg mangiferin equivalent per 1 g of dry weight.

Total flavonoid content was determined by a spectrophotometric method [28, 29] with rutin as a standard. Samples were air dried at room temperature to constant weight. We extracted 1] g of dry raw material using 70 % ethanol for 30 minutes in a flask equipped with a reverse cooler on a boiling water bath. The solution obtained was filtered. 1 ml of filtrate was placed into a 25 ml volume measuring flask, 5 ml of 2 % AlCl₃ solution in 95 % ethanol were added, and total volume was adjusted to 25 ml with 95 % (w:v) ethanol. Within 30 minutes, the optic density of the solution was measured with a spectrophotometer SPh-46 (410 nm).

The total flavonoid content was calculated from a calibration curve, and the result was expressed as the mg rutin equivalent per 1 g of dry weight.

The growth and biosynthetic characteristics of calli and root cultures were assessed on the

basis of dry weight outcome per 1 l of nutrient medium, as well as on the flavonoid and xanthone content in dry material obtained from 1 l of nutrient medium (further — flavonoids and xanthenes outcome from 1 l of nutrient medium). BAS (flavonoids or xanthenes) per 1 l of nutrient medium (X_3) was calculated according to the formula:

$$X_3 = \frac{m_{\text{dry}} \cdot X_1 \text{ or } X_2}{100}, \quad (3)$$

where m_{dry} — outcome of tissue culture or isolated root culture dry weight from 1 l of nutrient medium, g; X_1 — flavonoid total content in % of dry weight of raw material; X_2 — xanthone total content in % of dry weight of raw material.

ANOVA dispersion analysis with Tukey's HSD (honestly significant difference) test was performed with use of Prism 6. In checking statistical hypotheses of the research the critical level of significance was equal to 0.05.

Results and Discussion

Callus induction and proliferation

The frequency of callus induction (see Eq. 1) of most species on optimal media reached 100 %, excluding *G. acaulis* (70 %; Table 2).

In some species the efficiency of callus formation on the same culture medium depended on the genotype. For example, in the case of *G. asclepiadea* and *G. acaulis* the difference between the highest CI of two different genotypes was approximately 20 % (Table 2). *G. asclepiadea* callus induced on MS and MS/2 media underwent necrosis after 4-5 subcultures. We could obtain stable and highly proliferating callus using B₅ medium only

(Table 2). Callus induction was governed by the composition of MS culture medium. CI was up to 5 times higher on MS/2 than on standard MS medium in most samples.

The ratio and composition of PGR significantly affected the callus induction and proliferating activity. Callus obtained on MS with BAP and NAA had low CI (6–31 %) and short viability period. Only after one subculture multiple the necrosis in tissue was observed. The use of BAP (0.1 mg/l) and 2,4-D (0.5, 1 mg/l) stimulated the formation of pale yellow, friable callus with high proliferation potency. The highest callus induction frequency in most samples was obtained on the MS/2 medium supplemented with 0.1 mg/l BAP and 0.5 mg/l 2,4-D. However, in G.ac.T it was higher on the medium with 1 mg/l 2,4-D (Table 2). In the case of *G. asclepiadea* (G.asc.M genotype), the medium B₅ supplemented with 0.2 mg/l Kin stimulated CI more than 90 % (Table 2).

The MS/2 medium was optimal for callus proliferation of most species except for *G. asclepiadea* where the B₅ medium showed better results. We did not discover essential interspecies differences in the callus growth intensity (Fig. 2). The BAP and 2,4-D combination favored growth of all the cultures. Different genotypes of the same species efficiently proliferated in the same proliferation conditions (0.1 mg/l BAP and 0.5 mg/l 2,4-D). The active *G. asclepiadea* callus proliferation required double concentration of both cytokinin and auxin (0.2 mg/l BAP and 1 mg/l 2,4-D).

According to other authors, there is a lot of complexity in the callus initiation and proliferation for *Gentiana* species. It is supposed to be connected with a great number of phenolic

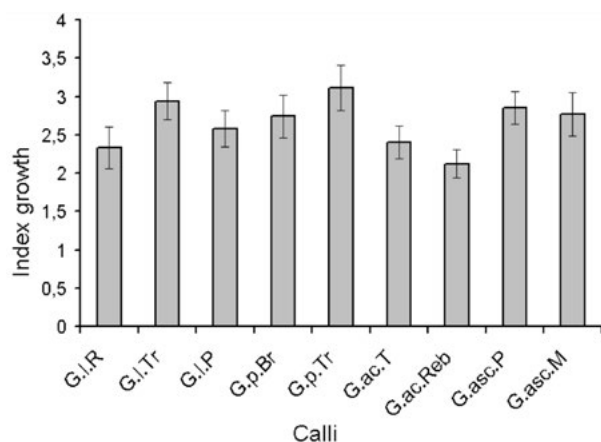


Fig. 2. Growth index of root origin calli of gentians on most effective nutrient media.

For notations, see Table 1

compounds in these plants [30]. For a rapid callus growth 2,4-D and BAP or Kin are usually used [30, 31].

In our study we obtained callus from 9 genotypes of 4 gentian species. BAP and 2,4-D growth regulators were necessary for both the callus induction and growth. The MS/2 medium supplemented with 0.1 mg/l BAP and 0.5-1 mg/l 2,4-D was found to be optimal for the callus induction and proliferation in three species. In the case of *G. asclepiadea* the callus formation required the B₅ medium supplemented with double concentration of both cytokinin and auxin (0.2 mg/l BAP and 1 mg/l 2,4-D).

Fast-growing root culture

MS/2 medium supplemented with 0.1 mg/l of BAP appeared to be the most effective for the isolated root formation and growth in *G. lutea*, *G. punctata* cultures at the first culture stage (Table 3).

Use of 0.1 mg/l of Kin was the best for the *G. acaulis* and *G. asclepiadea* cultures. The

samples from different geographic locations for two gentian species required the same concentrations of NAA: 0.3 mg/l for *G. asclepiadea*, 0.5 mg/l for *G. punctata*. However, for *G. lutea* efficient concentrations of NAA to form isolated roots constituted 1 or 2 mg/l, depending on genotype (Table 3). The intensive growth of *G. acaulis* isolated roots on MS/2 was significantly stimulated by doubled concentration of CaCl₂ (440 mg/l). In contrast to other species, *G. asclepiadea* produced isolated roots only on the B₅ medium supplemented with 0.1 mg/l Kin and 0.3 mg/l NAA (Table 3).

After the first culture stage the mean number of side rootlets per inoculum significantly varied: 84-101 for *G. lutea*, 95 for *G. acaulis*, 35-42 for *G. punctata* and 35-44 for *G. asclepiadea*. After the second culture stage the mean root size was the longest for *G. acaulis* and *G. lutea* (23 mm and 22.4-30.8 mm, respectively) and the shortest in *G. punctata* and *G. asclepiadea* (17.4-18.8 mm and 18.2-18.6 mm, respectively; Table 3).

Use of two-stage cultivation provided formation of a considerable number of side rootlets (in some cases over 100 per inoculum), an essential increase of side rootlets length (up to 30 mm) and their rapid growth (GI reached 926.5) (Table 3).

The highest yield of isolated roots (225 g of biomass per 1 l of medium) was obtained for the *G. lutea* cultures from Troyaska mountain samples. It is equal to the root mass of 10-12-year-old plant in nature [3]. GI of this culture was 926.5 and corresponded to GI obtained by other authors after the root transformation by *Agrobacterium rhizogenes*. For example, GI of nine clones of

Table 2. Frequency of callus induction (%) from root explants of four species on different media

Growth regulators, mg/l	<i>G. lutea</i>			<i>G. acutis</i>			<i>G. punctata</i>			<i>G. asclepiadea</i>	
	G.I.R ¹	G.I.Tr	G.I.P	G.ac.T	G.ac.Reb	G.p.Br	G.p.Tr	G.asc.P	G.asc.M		
BAP	6.3±0.4 ^a	7.8±0.4 ^a	8.3±0.6 ^b	8.5±0.5 ^a	9.3±0.6 ^a	23.8±1.1 ^a	23.8±2.1 ^a	28.9±2.6 ^a	31.3±2.6 ^a		
2	28.2±2.2 ^b	26.4±1.2 ^b	28.3±2.3 ^b	11.7±1 ^b	12.3±1 ^b	26.4±2.2 ^a	30.2±2.2 ^a	30.8±2.7 ^a	29.5±2.6 ^a		
BAP	100 ^{2c}	95.6±7.2 ^c	100 ^c	22.8±1.8 ^c	58.6±4.7 ^c	86.4±6.7 ^b	92.3±7.1 ^b	92.3±8.6 ^b	86.7±6.9 ^{bc}		
0.1	100 ^c	100 ^c	100 ^c	30.5±2.5 ^d	69.5±5.5 ^d	98.3±8.5 ^b	100 ^b	100 ^b	94.1±2.4 ^c		
0.1	42.5±2.9 ^d	39.2±3.1 ^d	40.4±3.1 ^d	29.4±2.2 ^d	31.2±2.4 ^e	13.5±1.1 ^c	15.5±1.1 ^c	60.2±4.3 ^c	57.3±5 ^d		
0.1	84.3±5.1 ^e	68.7±5.5 ^e	70.5±5.4 ^e	49.6±4.1 ^e	54.3±3.4 ^e	63.8±5.5 ^d	78.3±6.2 ^d	75.1±6.9 ^d	77.4±4.2 ^e		
0.2	— ³	—	—	—	—	—	—	61±4.9 ^{ce}	82.4±7.1 ^{bef}		
0.2	—	—	—	—	—	—	—	68.5±4.2 ^{de}	83.3±6.8 ^{bef}		
Kin	—	—	—	—	—	—	—	—	—		
2,4-D	—	—	—	—	—	—	—	—	—		
0.2	not tested	not tested	not tested	not tested	not tested	not tested	not tested	74±4.6 ^d	94.7±7.7 ^c		

¹ — Symbolic notation see in Table 1. ² — The highest values of callus induction efficiency are in the bold type. ³ — Callus induction was insignificant, planted explants underwent necrosis after 1-1.5 weeks of cultivation. ^{a, b, c, d, e, f} — the same Latin letters stand for unreliable difference of mean values for callus genesis from root explants of one genotype plants on different nutrient media according to Tukey's test.

Table 3. Some growth parameters for gentians isolated root culture at optimal concentrations of growth regulators

Genotype	Concentration of PGR ¹ in the medium (1 culture stage) ²	Mean number of side rootlets per inoculum	Mean length of side rootlets, mm		GPI ³ by fresh weight	Yield of biomass after 4-6 weeks of cultivation per 1l of medium, g
			I culture stage	II culture stage		
G.I.R ⁴	0.1 mg/l BAP +2 mg/l NAA	84.0±3.4 ^a	4.2±0.2 ^a	22.4±1.2 ^a	312.8 ±3.9 ^a	81.5±8.1 ^a
G.I.P	0.1 mg/l BAP +1 mg/l NAA	101.0±6.4 ^b	5.5±0.7 ^b	27.5±0.9 ^{ab}	428.0±9.3 ^b	85.6±3.8 ^a
G.I.Tr	0.1 mg/l BAP +0.5 mg/l NAA	98.0±8.4 ^b	6.3±0.7 ^b	30.8±5.9 ^b	926.5±7.6 ^c	225.5±7.2 ^b
G.p.Br	0.1 mg/l BAP +0.5 mg/l NAA	35±5.6 ^a	3.2±0.4 ^a	17.4±0.4 ^a	192.8±6.7 ^a	42.4±4.9 ^a
G.p.Tr	0.1 mg/l BAP +0.5 mg/l NAA	42±3.9 ^a	3.8±0.6 ^a	18.8±0.6 ^b	203.4±6.7 ^b	44.6±3.8 ^a
G.ac.T ⁵	0.1 mg/l Kin +0.5 mg/l NAA	95±5.7	4.2±0.5	23.06±5.3	324±4.8	71.28±4.8
G.asc.P	0.1 mg/l Kin +0.3 mg/l NAA	35.0±4.8 ^a	3.4±0.5 ^a	18.2±0.6 ^a	216.4±5.2 ^a	51.9±5.6 ^a
G.asc.M	0.1 mg/l Kin +0.3 mg/l NAA	44.0±6.2 ^b	3.8±0.5 ^a	18.6±0.5 ^a	250.2±7.3 ^b	55.0±6.6 ^a

¹ PGR — plant growth regulators. ² As basic was used MS/2 medium (except G.asc.P and G.asc.M cultivated on B₅ medium). ³ GI — growth index. ⁴ Symbolic notation see in Table 1. ⁵ The results are obtained on MS/2 medium with doubled concentration of CaCl₂. ^{a, b, c} — the same Latin letters stand for unreliable difference of mean growth values for genotypes of one species according to Tukey's test.

transformed *G. lutea* roots was 150.8-1473.2 [20].

The growth index of *G. punctata* roots of non-transgenic culture constituted 140.7, GI of transgenic — 45.3-287.7 for one strain and 33.8-1907.2 for the other one [21]. For the obtained by us non-transgenic culture of this species (G.p.Tr i G.p.Br) these values were 203.4 and 192.8 respectively. In the research of transgenic culture of the *Gentiana dinarica* Beck. isolated roots, the maximal GI reached 46.7 [32]. For the obtained by us *G. acaulis* culture — close to the *G. dinarica* species — this value equalled 324.

The yield of transgenic culture of the *Gentiana macrophylla* L. isolated roots was 130-260 g/l on the 30th day of cultivation [23]. The yield range of the investigated non-transgenic cultures of gentian isolated roots, obtained by us, was 42.4–225.5 g/l.

During the investigation of transgenic *G. punctata* roots, it was found that their length was 38.8 and 45.2 mm, whereas our results for non-transgenic culture of the same species constituted 17.4 and 18.8 mm. Thus, the root length of transgenic culture is bigger, whereas the number of side roots is considerably bigger in the obtained by us non-transgenic cultures (35 and 42 against 0 and 1.54). After 35 days the biomass of explants, cultivated on media with different concentration of sucrose, showed a 1.5-3.4 times increase [18].

Fast-growing root cultures of four *Gentiana* species plants were obtained and their growth was studied, in particular quantity and length of side rootlets, growth index and biomass production. The comparison of growth parameters of the obtained non-transgenic root cultures to those of the investigated transgenic

ones and other *Gentiana* species showed similar and in some cases even higher productivity indices of our cultures. We found that GI of the isolated root cultures was significantly higher (60-300 times) than GI of proliferating calli of the same species.

Xanthone and flavonoid contents in cultured tissues

The results of the analysis showed the diversity of total xanthone and flavonoid amounts in callus cultures of different species (Tables 4, 5).

A comparison of calli from *G. acaulis* and *G. lutea*, derived from plants from different localities, allowed establishing essential distinctions in the quantity of xanthone (Table 4). Thus, in the G.ac.T callus tissue, the amount of this class of compound exceeded 4.4 times an analogous value in the G.ac.Reb culture. G.l.R and G.l.Tr calli synthesized 2.6 and 2.5 times more xanthenes in comparison with the G.l.P culture. Xanthone content in calli was generally less than in shoots of intact plants (with the exception of *G. asclepiadea*), but greater than in roots. In the *G. punctata* cultures the amount of BAS was less than in roots (Table 4). Similar results were obtained for the *Gentianella austriaca* shoot cultures. It was shown by high-performance liquid chromatography, that the shoot cultures contained nearly two times less xanthenes than the plants growing in nature [33].

For nine tissue cultures, the highest value of total flavonoids was characteristic of G.ac.T (Table 5). This value was rather high in the *G. lutea* and G.ac.Reb calli. The *G. punctata* and *G. acaulis* calli derived from plants of various localities, differed considerably by the

amount of flavonoids (Table 5). In the G.ac.T callus there were 1.8 times more flavonoids compared to the G.ac.Reb culture. Flavonoid content in all tissue cultures was considerably less than in shoots of intact plants. In some calli, namely G.p.Tr, G.l.Tr and G.l.P, the amount of these BAS exceeded that in roots (Table 5).

Table 4. Total xanthone contents in different organs of intact gentian plants, calli and isolated cultured roots.

Samples	Total xanthone contents, mg MAN/g DW ¹			
	Shoots	Roots	Callus	Isolated root cultures
G.l.P ²	28.4±1.5 ^{ad}	4.7±0.4 ^{ab}	3.8±0.1 ^{ae}	14.8±1.2 ^a
G.l.Tr	19.9±1.2 ^b	4.3±0.2 ^a	9.5±0.6 ^b	12.3±1.1 ^b
G.l.R	39.2±2.1 ^c	5.5±0.4 ^b	9.8±0.8 ^b	11.8±0.9 ^b
G.p.Tr	27.3±1.7 ^a	26.1±1.9 ^c	3.2±0.2 ^a	5.0±0.3 ^c
G.p.Br	34.2±2.3 ^{cd}	32.9±2.8 ^c	5.0±0.4 ^c	5.8±0.3 ^{cd}
G.ac.T	21.1±1.1 ^b	7.4±0.4 ^d	9.2±0.7 ^b	12.1±0.8 ^b
G.ac.Reb	27.8±2.4 ^a	2.9±0.2 ^e	2.1±0.2 ^d	not determined
G.asc.P	4.2±0.3 ^e	0.8±0.05 ^f	4.4±0.3 ^{ce}	6.0±0.4 ^d
G.asc.M	5.3±0.4 ^f	0.9±0.07 ^f	6.4±0.5 ^f	not determined

¹ mg mangiferin equivalent (MAN)/g dry weight (DW).

² For notations see Table 1.

a, b, c, d, e, f — the same Latin letters stand for unreliable difference of mean values for xanthones content in corresponding samples of different genotypes according to Tukey's test.

Table 5. Total flavonoid contents in different organs of intact gentian plants, calli and isolated cultured roots.

Samples	Total flavonoid content, mg (RUT)/g DW ²			
	Shoots	Roots	Callus	Isolated root cultures
G.l.P ¹	55.2±4.2 ^a	4.4±0.3 ^a	5.3±0.4 ^a	6.5±0.4 ^a
G.l.Tr	35.3±2.9 ^b	4.6±0.2 ^a	6.2±0.6 ^b	8.4±0.6 ^b
G.l.R	68.3±6.2 ^c	4.8±0.3 ^a	4.9±0.4 ^a	5.3±0.3 ^c
G.p.Tr	66.4±5.8 ^c	1.4±0.8 ^b	2.8±0.2 ^c	6.2±0.3 ^a
G.p.Br	62.8±5.4 ^{ac}	3.4±0.3 ^c	3.3±0.3 ^c	9.4±0.6 ^d
G.ac.T	42.8±3.2 ^b	12.3±1.0 ^d	13.1±0.9 ^d	14.3±1.0 ^e
G.ac.Reb	20.2±1.9 ^d	16.2±4.7 ^e	7.1±0.7 ^e	not determined
G.asc.P	26.5±2.1 ^d	3.5±0.3 ^c	3.2±0.3 ^c	9.0±0.7 ^{bd}
G.asc.M	21.2±1.6 ^d	2.3±0.2 ^f	2.1±0.2 ^f	not determined

¹ For notations see Table 1.

² mg rutin equivalent (RUT)/g dry weight (DW).

a, b, c, d, e, f — the same Latin letters stand for unreliable difference of mean values for flavonoids content in corresponding samples of different genotypes according to Tukey's test.

Dry weight outcome after four weeks of callus growth varied within 11.6 to 29.3 g/l (Fig. 3). This value was the highest for the G.ac.Reb tissue cultures, and the lowest for *G. asclepiadea*. Assessment of the biosynthetic characteristics of gentian calli showed their ability to accumulate from 50 to 249 mg of xanthenes per 1 l of nutrient medium (see Eq. 3). This value was the highest for the G.l.R, G.l.Tr and G.ac.T tissue cultures, and the lowest — in the G.asc.P callus (Fig. 3). The gentian tissue cultures are able to accumulate 37–279 mg of flavonoids per 1 l of cultural medium (see Eq. 3). This value was the greatest in the *G. acaulis* calli (Fig. 3), but lowest in the tissue cultures of *G. asclepiadea*. The given data prove that in most callus cultures, the growth conditions favoured both biomass production and BAS (xanthenes and flavonoids) synthesis. However, in some cases, in the G.asc.P callus for example, low values were characteristic of dry biomass and BAS.

Xanthone and flavonoid content in the isolated root cultures

The root cultures obtained from *G. lutea*, *G. punctata*, *G. acaulis* and *G. asclepiadea* synthesize large amount of flavonoids and xanthenes as compared to calli (Tables 4, 5). The greatest flavonoid content (14.3 mg (RUT)/g DW) was found in the *G. acaulis* root cultures; the highest values of xanthenes (12.1–14.8 mg (MAN)/g DW) were characteristic for the isolated roots of *G. lutea* and *G. acaulis*.

Comparison of root cultures derived from plants from different localities showed that there were no significant distinctions of BAS content for *G. lutea* and *G. punctata* (Tables 4, 5).

It is shown that in isolated root cultures of *G. lutea* and *G. acaulis* the amount of xanthenes was smaller compared to shoots, but larger than in roots. The amount of xanthenes in the culture of *G. punctata* isolated roots was

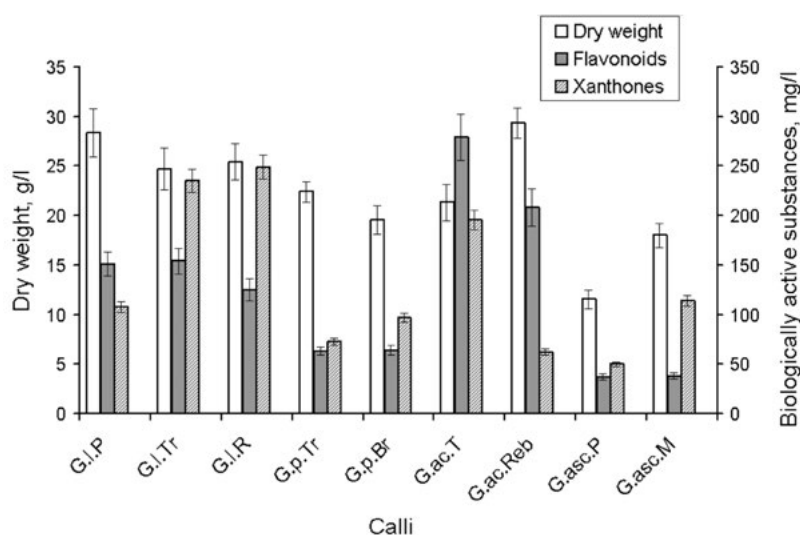


Fig. 3. Productivity of gentian tissue cultures by dry weight and biologically active substances (flavonoid and xanthone) per 1 l of nutrient medium.

For notations, see Table 1

smaller than in both roots and shoots of the plants from natural populations (Table 4).

In all root cultures investigated the amount of flavonoids was considerably smaller than in shoots, but larger or practically the same as in roots (Table 5).

Similar results were obtained for extracts from the *Withania somnifera* Dunal isolated cultured roots. It was shown by high-performance liquid chromatography that the content of alkaloid withanolides in cultured roots constituted 4 mg/g of their dry weight and exceeded the analogous parameter value in roots *in vivo* (3 mg/g of dry weight) [34].

After 4-6 weeks of growth the dry weight of isolated cultured roots varied from 7.3 to 34.5 g/l (Fig. 4). This parameter value was higher for G.I.Tr roots in culture. The least isolated root growth was characteristic of *G. punctata*.

The isolated root cultures are characterized by their capacity to accumulate 50.3–318.2 mg of flavonoids and 40.6–424.3 mg of xanthenes

per 1 l of nutrient medium (see Eq. 3). The productivity values by both BAS amounts were the highest for the G.I.Tr cultured roots, when comparing them with rather high values for the G.ac.T, G.I.P and G.I.R isolated roots (Fig. 4).

Thus, the growth conditions for most of isolated root cultures favoured the BAS synthesis in biomass accumulation. However, for the *G. punctata* cultures both these parameters were low.

Generalization of the results concerning flavonoid and xanhone accumulation in gentian calli and isolated root cultures showed the following: 1) most of gentian cultures are able to accumulate flavonoids and xanthenes in amounts, which in some cases reached 1.5 % of dry weight; 2) in calli tissues and isolated root cultures, the amount of BAS was greater or close to that in roots of plants, but considerably less than in shoots; 3) in most of the isolated root cultures analyzed there were more flavonoids and xanthenes than in calli, although the difference of the BAS content be-

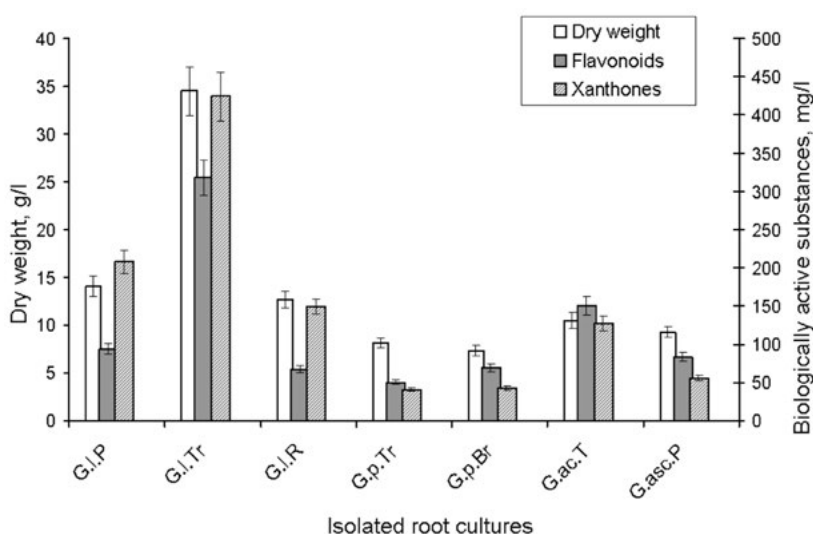


Fig. 4. Productivity of the gentian isolated root cultures by dry weight and biologically active substances per 1 l of nutrient medium.

For notations, see Table 1

tween the two types of cultures was insignificant; 4) BAS amount in both morphogenic and non-morphogenic cultures depended on the genotype of the plant donor; 5) a correlation was found between flavonoid and xanthone content *in vitro*.

Growth conditions enable the biomass accumulation as well as the flavonoid and xanthone synthesis in callus and isolated root cultures. The above growth and biosynthesis parameters of cultures prove that gentians are potential material as a source for obtaining valuable secondary metabolites. In general, the essential distinctions were not found for biomass and BAS per 1 l of nutrient medium between callus tissues and corresponding root cultures. However, for the latter we established high growth index values (192-926) that exceeded by 60-300 times those for callus cultures. Obtaining and growing isolated roots are less labour-consuming and methodically less complicated than initiating and maintaining callus. To gain biomass and BAS synthesis for isolated roots 4-6 weeks are required, whereas for stable tissue cultures 11-15 passages are necessary, each passage lasting 4 weeks. Isolated root can be cultured in liquid nutrient media without agar or growth regulators at the second stage of culture. All these factors reduce the material costs for the isolated root cultures and improve their availability in comparison to callus tissues.

Conclusion

In vitro tissue and organs cultures of *Gentiana* species from the Ukrainian flora were obtained. The capacity to form morphogenic and non-morphogenic cultures and their growth depended on the original genotype, mineral

composition and PGR content of nutrient medium. In some cases the efficiency of callus formation from root and stem explants rated up to 100 %. The highest GI for calli from root explants was within 2.1-3.1. The fast-growing root cultures were characterized by a high growth rate (growth index reached 927) and considerable yield of biomass.

The tissue and fast-growing root cultures are able to synthesize flavonoids and xanthenes in amounts which in some cases reached 1.5 mg/g of dry weight. The amount of these BAS in calli and isolated roots was considerably less than in shoots of plants, but more or approximately the same as in roots. The number of secondary metabolites investigated varied depending on the original plant genotype and culture type. The greatest productivity was in both types of *G. lutea* and *G. acaulis* cultures. Taking into consideration the outcome of gentian tissue cultures and roots as well as their capacity to synthesize flavonoids and xanthenes, they have a great potential as an alternative source of these BAS. Compared to callus tissues, a preference should be given to the isolated root cultures that are characterized by a considerably higher growth index and less complicated growth pattern.

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Отримання і аналіз культури тканин та швидкорослої культури коренів чотирьох рідкісних високогірних лікарських видів роду *Gentiana* L.

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Мета. Отримання культури тканин та ізольованих коренів чотирьох видів *Gentiana* L. флори України (*G. lutea* L., *G. punctata* L., *G. acaulis* L. і *G. asclepiadea* L.), а також дослідження особливостей їхнього росту і вмісту флавоноїдів та ксантонів. **Методи.** Культивування *in vitro*, хроматоспектрофотометричні і статистичні методи. **Результати.** Розроблені умови для індукції і проліферації калюсів, а також тривалого культивування швидкорослих культур коренів тирличів. Проведено порівняльні дослідження загального вмісту флавоноїдів і ксантонів у калюсах, культурах ізольованих коренів та дикорослих рослинах тирличів. Встановлено здатність культур до синтезу цих біологічно активних речовин *in vitro*. Кількість таких сполук варіювала як у калюсах і культурах ізольованих коренів, отриманих з рослин різних видів тирличів, так і в культурах тканин й органів, отриманих з різних рослин одного виду. Вміст флавоноїдів і ксантонів у морфогенних і неморфогенних культурах був значно нижчий, ніж у пагонах, але більший або близький до такого в коренях інтактних рослин. Швидкоросла культура

коренів у більшості випадків характеризувалася вищою концентрацією цих біологічно активних сполук, ніж каллус. **Висновок.** Високий вихід біомаси культур *in vitro* тирличів та їх здатність синтезувати і накопичувати флавоноїди та ксантони дозволяють розглядати їх як перспективне джерело цих біологічно активних сполук.

Ключові слова: види роду *Gentiana* L.; індукція та проліферація каллусу; швидкоросла культура коренів; вміст флавоноїдів і ксантонів; джерело біологічно активних сполук.

Получение и анализ культуры тканей и быстрорастущей культуры корней четырех редких высокогорных лекарственных видов *Gentiana* L.

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Цель. Получение культуры тканей и изолированных корней четырех видов *Gentiana* L. флоры Украины (*G. lutea* L., *G. punctata* L., *G. acaulis* L. и *G. asclepiadea* L.), а также исследование особенностей их роста и содержания флавоноидов и ксантонов.

Методы. Культивирование *in vitro*, хроматоспектрофотометрические и статистические методы.

Результаты. Разработаны условия для индукции и пролиферации каллусов, а также длительного культивирования

вирувания быстрорастущих культур корней горечавок. Проведены сравнительные исследования общего содержания флавоноидов и ксантонов в каллусах, культурах изолированных корней и дикорастущих растениях горечавок. Установлена способность культур к синтезу этих биологически активных веществ *in vitro*. Количество таких соединений варьировало как в каллусах и культурах изолированных корней, полученных из растений различных видов горечавки, так и в культурах тканей и органов, полученных из различных растений одного вида. Содержание флавоноидов и ксантонов в морфогенных и неморфогенных культурах было значительно ниже, чем в побегах, но больше или близкое к таковому в корнях интактных растений. Быстрорастущая культура корней в большинстве случаев характеризовалась высшей концентрацией этих биологически активных соединений, чем каллус.

Вывод. Высокий выход биомассы культур *in vitro* горечавок, а также их способность синтезировать и накапливать флавоноиды и ксантоны, позволяют рассматривать их как перспективный источник этих биологически активных веществ.

Ключевые слова: виды рода *Gentiana* L.; индукция и пролиферация каллуса; быстрорастущая культура корней; содержание флавоноидов и ксантонов; источник биологически активных веществ.

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