

## Chapter 2

# In Vitro Manipulation and Propagation of *Gentiana* L. Species from the Ukrainian Flora

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**Abstract** Conditions were developed for microclonal propagation, callus induction and proliferation, plant regeneration, and long-term maintenance of fast-growing normal root cultures of *Gentiana* species from the Ukrainian flora. The basic growth parameters were evaluated for cultured tissues. Extensive growth and considerable biomass yield was achieved in most cultures. The ability to form tissue and organ cultures depended on the original genotype, type of explant, growth regulator, and mineral composition of the nutrient medium. The efficiency of regeneration from cultured tissue declined with the duration of callus maintenance.

## 2.1 Introduction

The genus *Gentiana* is represented by 10 species in the Ukraine, namely 8 perennial (*Gentiana acaulis* L., *Gentiana asclepiadea* L., *Gentiana cruciata* L., *Gentiana laciniata* Kit. ex Kanitz., *Gentiana lutea* L., *Gentiana pneumonanthe* L., *Gentiana punctata* L., *Gentiana verna* L.) and 2 annuals (*Gentiana nivalis* L. and *Gentiana utriculosa* L.) (Zerov 1957; Strashniuk et al. 2005). Most of them (with the exception of *G. pneumonanthe* and *G. cruciata*) are spread throughout the Carpathians. Seven species are registered in the Red Book of Ukraine, these being *G. nivalis*, *G. utriculosa* and *G. verna* which are endangered, *G. acaulis* and *G. laciniata* which

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are rare, and *G. lutea* and *G. punctata* that are vulnerable to loss (Didukh 2009). Such a catastrophic situation is caused by both reduction of rare gentian species areas as a result of natural habitat destruction, and populations decrease because of unregulated collecting of raw material of these valuable medicinal plants.

Cultivation of these species in situ is problematic, taking into consideration their biological peculiarities. In particular, gentians are characterized by low seed germination, late flowering, soils and climatic conditions requirements, the need for specific pollinators and mycorrhizal fungi in the soil, as well as slow biomass accumulation. For example to obtain 100–200 g of rhizome it is necessary to grow plants for 10–12 years. Therefore, to restore natural gentian populations, stability and replenishment of raw material sources alongside traditional methods use necessary together with modern biotechnological approaches.

The results of investigations of *Gentiana* species of the Ukrainian flora are presented in this chapter.

## 2.2 Plant Material and in vitro Techniques

Plants of seven *Gentiana* species were obtained from seeds harvested in different habitats (Table 2.1), and grown in aseptic conditions in vitro.

### 2.2.1 Microclonal Propagation

Microclonal propagation employed plants of sixteen genotypes of seven gentian species. Stem cuttings (shoots with axillary buds) of 2- to 3-month-old axenic plants were used as initial explants. Both liquid and semi-solid MS-based medium (Murashige and Skoog 1962) with decreased macro- and microsalts concentrations (MS/2), and altered CaCl<sub>2</sub> concentrations (220 and 440 mg/l) were used. Increased CaCl<sub>2</sub> concentration was used, taking into consideration data concerning the calciphilous nature of some *Gentiana* species (Biront et al. 1993). Medium was supplemented with the cytokinins 6-benzylaminopurine (BAP) and kinetin (Kin).

Assessment of microclonal propagation was after 1–2 months by determining multiple shoot induction (MSI) and the mean number of shoots per explant (MNS). MSI was calculated according to the formula:

$$MSI = \frac{N_s}{N} \times 100 \%, \quad (2.1)$$

where  $N_s$ —number of explants on which shoots formed;  $N$ —number of cultured explants.

**Table 2.1** Habitat of the investigated gentians

Species	Locality	Altitude (m)	Symbol
<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
	Pozhyzhevska mountain (Chornohora range, Nadvirna district, Ivano-Frankivsk region)	1420	G.l.P
	Lemska mountain valley (Chornohora range, Rakhiv district, Transcarpathian region)	1500	G.l.L
	Rivna mountain valley (Polonyna range, Perechyn district, Transcarpathian region)	1400	G.l.Riv
<i>G. punctata</i>	Pozhyzhevska mountain, (Chornohora range, Nadvirna district, Ivano-Frankivsk region)	1480	G.p.P
	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
	Brebeneskul mountain (Chornohora range, Rakhiv district, Transcarpathian region)	1940	G.ac.Bre
<i>G. asclepiadea</i>	Pozhyzhevska 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
<i>G. cruciata</i>	Village of Krenychi (Obukhiv district, Kyiv region)	—	G.cr.Kr
	“Medobory” nature reserve (Husiatyn district, Ternopil region)	—	G.cr.Med
<i>G. pneumonanthe</i>	Koriukivka forestry (Koriukivka district, Chernihiv region)	—	G.pn.K
	Village of Vyhoda (Dolyna district, Ivano-Frankivsk region)	450–500	G.pn.V
<i>G. verna</i>	Heredzhivka hole (village of Yasynia, Rakhiv district, Transcarpathian region)	750–800	G.v.G

MNS was determined according to the formula:

$$\text{MNS} = \frac{S}{N}, \quad (2.2)$$

where  $S$ —total number of formed shoots;  $N$ —number of cultured explants.

Microclones were separated from initial cuttings and enrooted on the nutrient MS/2 medium, supplemented with 0.1 mg/l 1-naphthaleneacetic acid (NAA) (3–4 weeks). Rooted shoots were transferred into MS/2 medium without plant growth regulators (PGR) and sucrose for 10–14 days. Plants were washed from the remains of agar and transferred into soil from natural habitats. Some plants (5–20 % from the number of rooted in vitro) were characterized by delayed growth and lower viability and therefore were not used for transfer to soil. To adapt plants to ex vitro condition, the plants were kept under conditions of increased humidity (65–80 %) and decreased lighting intensity (1000–1500 lux) for 5–10 days.

### 2.2.2 Callus Induction and Proliferation

Leaf, stem, and root explants of 16 axenic genotypes of 7 *Gentiana* species (Table 2.1) were used. Every each of experiment comprised of testing 100–150 explants. Media used for callus induction (CI) and proliferation were B<sub>5</sub> (Gamborg and Eveleigh 1968), MS, and MS/2, supplemented with various combinations and concentrations of Kin, BAP, 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA. Cultures were incubated in darkness under the temperature at 25–26.5 °C, with subculture every 4 weeks. The percentage of CI was recorded after 3 weeks of culture. CI frequency was determined as follows:

$$CI = \frac{N_c}{N} \times 100 \%, \quad (2.3)$$

where  $N_c$ —number of explants with callus, and  $N$ —number of explants cultured.

In order to determine the optimal conditions for callus proliferation, 0.25–0.35 g portions of callus were excised from explants and placed on medium with various combinations of BAP and 2,4-D.

The growth index (GI) according to callus fresh weight was determined after 3 weeks according to the formula:

$$GI = \frac{M - m}{m}, \quad (2.4)$$

where  $M$ —callus weight after 3 weeks;  $m$ —initial callus weight.

### 2.2.3 Direct Organogenesis

Experiments were carried out on axenic cultures of *G. lutea* from the localities of Troyaska, Rohneska, Lemska, and Pozhyzhevsk. Experiments were initiated using leaf (area 1–1.5 cm<sup>2</sup>), stem, and root (5 mm long) explants on MS medium

supplemented with different concentrations of thidiazuron (TDZ) (1, 5, 10, 20 mg/l) and NAA (0.01, 1 mg/l). Regeneration was determined after 1.5–2 months using the indices regeneration percentage (RP) and mean number of regenerants per explant with regenerants (MNR).

RP was calculated by the formula:

$$RP = \frac{Nr}{N} \times 100 \%, \quad (2.5)$$

where  $Nr$ —the number of explants developing shoots or roots;  $N$ —the number of cultured explants.

MNR was determined by the formula:

$$MNR = \frac{R}{Nr}, \quad (2.6)$$

where  $R$ —the number of regenerants;  $Nr$ —the number of explants developing shoots or roots.

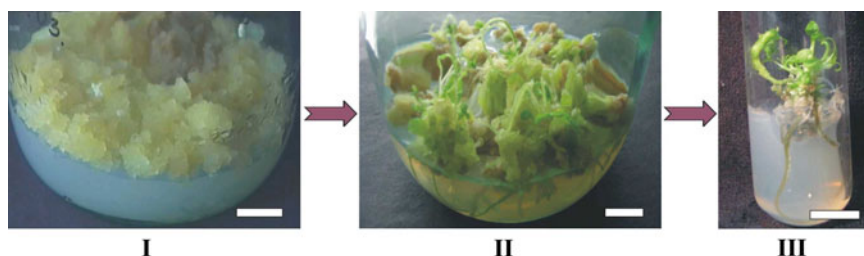
Estimation of regeneration efficiency (RE) was after 1.5–2 months and calculated from formula:

$$RE = \frac{R}{N}, \quad (2.7)$$

where  $R$ —the number of regenerants;  $N$ —the number of explants cultured.

### 2.2.4 Plant Regeneration from Callus

Experiments employed MS medium supplemented with combinations and concentrations of PGRs: (1) TDZ (1, 5, 10, 20 mg/l) and NAA (0.01, 0.1, 0.2, 0.5, 1 mg/l); (2) BAP (0.1, 0.2, 0.5 mg/l) and NAA (1, 1.5, 2, 3, 4, 5 mg/l). The experiment was conducted according to the scheme shown in Fig. 2.1 with three



**Fig. 2.1** Scheme of induction of indirect regeneration. *I* Non-morphogenic callus proliferation. *II* Morphogenic tissue. *III* Roots and shoots regeneration. Bars = 1 cm

replicates. Callus samples were cultured in Petri dishes, with 15–20 samples per plate. The number of samples tested for each variant of the experiment was 50–60.

Regenerated efficiency was assessed by regeneration percentage (PR; see Eq. 2.5) of shoots or roots, mean number of shoots per explant with shoots (MNS; see Eq. 2.2) and mean number of roots per explant with roots (MNRhiz) according to the Eq. 2.6, where R – the number of roots; Nr – the number of explants developing roots.

For rooting the regenerants, MS and MS/2 media with various concentrations of BAP or Kin and gibberellic acid ( $GA_3$ ) or NAA were used, as well as media lacking growth regulators.

Regenerants were rooted on hormone-free MS and MS/2 media or with various concentrations of selected PGRs [BAP, Kin, and gibberellic acid ( $GA_3$ ) or NAA].

### 2.2.5 Fast-Growing Root Culture

Root cultures were established for different genotypes of six *Gentiana* species shown in Table 2.1. Root apices each 1.5 cm long and 20–30 mg were used as initial explants for obtaining isolated root cultures, inocula being taken from two-month axenic plants. Root cultures were obtained in two stages according to the scheme in Fig. 2.2. At the first stage, the inocula were cultured for 2–3 weeks in MS/2 or  $B_5$  liquid nutrient medium, supplemented with various concentrations of NAA and BAP or Kin. At the second stage, cultured roots with side rootlets were cultured in MS/2 or  $B_5$  liquid medium without growth regulators for 2–3 weeks. The pH of both media was  $5.6 \pm 0.2$  before autoclaving. Both cultures required two- to three-week period. The cultures were held in 250-ml Erlenmeyer flasks each with 50 ml of medium. The culture maintenance was in darkness, with constant stirring at  $60\text{--}80$  rpm at  $24 \pm 2$  °C.



**Fig. 2.2** Scheme for obtaining gentians isolated root cultures. 0 Selection of inocula (root apices of 1–1.5 cm long) of 2 month axenic plants. I Initiation of the formation and growth of side rootlets on the MS/2 medium with 0.1 mg/l BAP/Kin and 0.3–2 mg/l NAA. II Root biomass growth on MS/2 medium without growth regulators. Bars = 1 cm

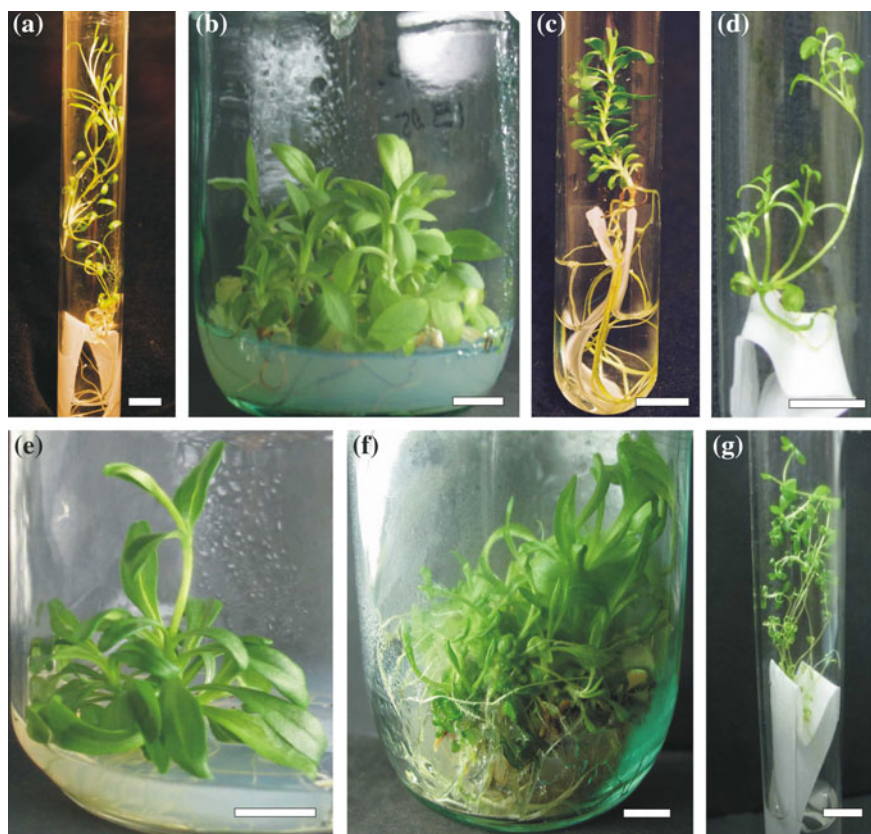
The inocula before placing in the medium and isolated root cultures after 4–6 weeks of culture (the first and second culture stages) were weighed in axenic conditions with further determination of their growth index by fresh weight according to the Eq. 2.4, where  $M$ —isolated root weight after 4–6 weeks of culture, and  $m$ —initial inoculum weight. In addition to growth index, the mean number of side rootlets per cultured inocula were determined as well as mean length after the first and second stages of culture. These parameters are important to characterise isolated root cultures, as they indicate the capability of roots to grow continuously (Kalinin et al. 1980). 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. Results were processed statistically (Lakin 1980).

## 2.3 Microclonal Propagation

### 2.3.1 Influence of Exogenic Growth Regulators and Calcium

The formation of microclones occurred in liquid and on semi-solid agar media (Fig. 2.3). Microclones formation on explants of the 7 genotypes of the 4 species investigated—*G. acaulis*, *G. cruciata*, *G. punctata* and *G. asclepiadea*—was most effective on medium with 0.5 mg/l BAP and 0.1 mg/l Kin (Tables 2.2, 2.3, and 2.4). MSI (see Eq. 2.1) was within 69.4–90.1 % and MNS (see Eq. 2.2) being 2.6–5.9. A 10 times decrease of BAP concentration (to 0.05 mg/l) was most favorable for *G. lutea* shoot formation (Table 2.2). MSI was within 74.5–91.7 %; MNS—4.3–6.5. Supplementing medium with 0.2 mg/l BAP and 0.2 mg/l Kin induced efficient micropropagation of *G. pneumonanthe* and *G. acaulis* plants (G.ac.Reb) (MSI—66.8–81.2 %, MNS—3.1–7.2) (Tables 2.2, 2.3, and 2.4). The use of nutrient medium with increased up to 1 mg/l content of BAP and 0.2 mg/l Kin was optimal for *G. cruciata* (G.cr.Kr) and *G. verna*: MSI—62.3 and 74.5 %, MNS—6.1 and 7.4, respectively (Table 2.4).

Microclonal propagation efficiency of *G. lutea* and *G. acaulis* with growth regulators was influenced substantially by increase in calcium. In particular, nutrient media with the same content of BAP and Kin but twice  $\text{CaCl}_2$  resulted in much greater values for micropropagation for *G. lutea* plants from the Rivna mountain valley (MSI—91.7 %; MNS—4.3) and *G. acaulis* from the Turkul mountain (MSI—83.3; MNS—2.6). In contrast, such increase of  $\text{CaCl}_2$  reduced MSI and MNS of *G. lutea* and *G. acaulis* from other geographic locations (Table 2.2).



**Fig. 2.3** Microclonal propagation of some *Gentiana* species. **a** *G. lutea*, **b** *G. punctata*, **c** *G. acaulis*, **d** *G. asclepiadea*, **e** *G. pneumonanthe*, **f** *G. cruciata*, **g** *G. verna*. Bars = 1 cm

### 2.3.2 Dependence on Species and Genotype

Values of microclone formation for plants of 7 species differed substantially. The optimum multiplication capacity was for *G. verna*, with MNS having the greatest value in the sampling (7.5). However, for *G. punctata* and *G. acaulis*, these values were the lowest and were only 2.6–3.2 and 2.6–3.1, respectively.

Plant-donor genotype influence on the capacity to form shoots for *G. acaulis* and *G. cruciata*. Thus, for *G. acaulis* plants from the Turkul and Rebra mountains, optimum medium differed not only by the concentrations of BAP and Kin, but also in  $\text{CaCl}_2$  content (Table 2.2). Multiplication efficiency of *G. cruciata* plants from Krenychi village was stimulated by nutrient medium with 1 mg/l BAP and 0.2 mg/l Kin, and plants from “Medobory” natural reserve by twice reduced concentrations of both cytokinins (Table 2.4).



**Table 2.2** Effect of cytokinins on multiple shoot induction in different genotypes of *G. lutea* and *G. acaulis*

Growth regulators (mg/l)		<i>G. lutea</i>				<i>G. acaulis</i>			
		CaCl <sub>2</sub>		G.l.Tr		G.l.Riv		G.l.P	
		G.l.R <sup>a</sup>	MST <sup>c</sup> , %	MNS	MSI, %	MNS	MSI, %	MNS	MSI, %
BAP	Kin								
0.05	0.1	N <sup>d</sup>	<b>5.50<sup>f</sup> ± 0.45</b>	<b>5.88 ± 0.55</b>	<b>82.3 ± 6.78</b>	1.5 ± 0.11	54.2 ± 5.17	<b>6.5 ± 0.36</b>	<b>82.7 ± 7.34</b>
		2N <sup>e</sup>	2.28 ± 0.21	2.95 ± 0.23	61.4 ± 5.98	<b>4.25 ± 0.41</b>	<b>91.7 ± 5.63</b>	1.2 ± 0.09	35.4 ± 3.18
0.5	0.1	N	1.14 ± 0.09	1.32 ± 0.08	45.7 ± 3.87	0.46 ± 0.03	31.4 ± 2.58	1.34 ± 0.11	42.4 ± 3.48
		2N	1.47 ± 0.13	2.04 ± 0.18	59.2 ± 5.33	1.85 ± 0.12	56.8 ± 5.15	0.9 ± 0.06	30.1 ± 2.58
0.2	0.2	N	1.48 ± 0.19	1.66 ± 0.21	49.4 ± 4.18	1.16 ± 0.08	39.7 ± 3.38	1.85 ± 0.16	64.1 ± 5.11

<sup>a</sup>Symbol notation, see Table 2.1

<sup>b</sup>MNS mean number of shoots per explant

<sup>c</sup>MST multiple shoot induction

<sup>d</sup>N CaCl<sub>2</sub> content in the nutrient medium 220 mg/l

<sup>e</sup>2N CaCl<sub>2</sub> content in the nutrient medium 440 mg/l

<sup>f</sup>The largest values of micropropagation efficiency are in bold type

**81.2 ± 6.79**

**83.3 ± 7.61**

**2.63 ± 0.24**

**3.1 ± 0.27**

**81.2 ± 6.79**

**83.3 ± 7.61**

**2.63 ± 0.24**

**3.1 ± 0.27**

**Table 2.3** Effect of cytokinins on multiple shoot induction in different genotypes of *G. punctata* and *G. asclepiadea*

Growth regulators (mg/l)		<i>G. punctata</i>			<i>G. asclepiadea</i>					
		CaCl <sub>2</sub>	G.p.Br <sup>a</sup>		G.p.Tr		G.p.P		G.asc.P	
			MNS <sup>b</sup>	MSI <sup>c</sup> , %	MNS	MSI, %	MNS	MSI, %	MNS	MSI, %
BAP	Kin									
0.05	0.1	N <sup>d</sup>	2.28 ± 0.17	68.2 ± 4.76	2.47 ± 0.21	73.4 ± 6.18	1.52 ± 0.07	44.5 ± 4.12	1.44 ± 0.03	54.6 ± 4.18
		2N <sup>e</sup>	1.12 ± 0.09	49.4 ± 3.76	0.68 ± 0.04	28.4 ± 3.36	0.68 ± 0.03	22.8 ± 2.16	0.85 ± 0.07	32.2 ± 2.94
0.5	0.1	N	<b>3.11<sup>f</sup> ± 0.24</b>	<b>69.5 ± 5.54</b>	<b>3.22 ± 0.19</b>	<b>80.3 ± 6.98</b>	<b>2.62 ± 0.19</b>	<b>74.9 ± 7.11</b>	<b>4.60 ± 0.31</b>	<b>89.4 ± 7.87</b>
		2N	1.23 ± 0.08	44.4 ± 3.26	1.48 ± 0.12	39.8 ± 2.84	0.8 ± 0.06	36.7 ± 3.24	1.1 ± 0.07	48.6 ± 3.48
0.2	0.2	N	2.46 ± 0.22	58.2 ± 4.18	2.88 ± 0.27	76.4 ± 6.14	2.35 ± 0.22	70.2 ± 6.98	2.82 ± 0.21	64.6 ± 4.28

<sup>a</sup>Symbol notation, see Table 2.1

<sup>b</sup>MNS mean number of shoots per explant

<sup>c</sup>MSI multiple shoot induction

<sup>d</sup>N CaCl<sub>2</sub> content in the nutrient medium 220 mg/l

<sup>e</sup>2N CaCl<sub>2</sub> content in the nutrient medium 440 mg/l

<sup>f</sup>The largest values of micropropagation efficiency are in bold type

**Table 2.4** Effect of cytokinins on multiple shoot induction in different genotypes of *G. cruciata*, *G. pneumonanthe*, and *G. verna*

Growth regulators (mg/l)		CaCl <sub>2</sub>	<i>G. cruciata</i>			<i>G. pneumonanthe</i>			<i>G. verna</i>			
			G.cr.Kr <sup>a</sup>		G.cr.Med		G.pn.K		G.pn.V		G.v.G	
			MNS <sup>b</sup>	MSI <sup>c</sup> , %	MNS	MSI, %	MNS	MSI, %	MNS	MSI, %	MNS	MSI, %
BAP	0.05	N <sup>d</sup>	0.44 ± 0.02	34.4 ± 3.16	0.78 ± 0.04	19.2 ± 1.86	0.95 ± 0.04	24.2 ± 2.28	1.45 ± 0.09	22.4 ± 2.14	1.44 ± 0.12	66.4 ± 5.76
	0.1		0.89 ± 0.05	40.2 ± 4.18	1.78 ± 0.12	29.8 ± 2.16	5.21 ± 0.32	52.8 ± 5.39	6.11 ± 0.53	56.4 ± 4.76	1.88 ± 0.14	68.2 ± 5.88
	0.5	N	6.22 ± 0.21	48.2 ± 3.34	<b>5.94<sup>f</sup> ± 0.36</b>	<b>69.4 ± 7.44</b>	1.86 ± 0.13	38.3 ± 2.98	2.32 ± 0.18	32.2 ± 3.12	2.22 ± 0.18	72.2 ± 6.44
	0.2	2N	1.64 ± 0.16	34.8 ± 3.36	3.84 ± 0.18	43.8 ± 4.13	<b>6.32 ± 0.43</b>	<b>74.7 ± 6.34</b>	<b>7.21 ± 0.64</b>	<b>66.8 ± 6.86</b>	3.11 ± 0.24	80.2 ± 7.34
1	0.2	N	<b>7.35 ± 0.28</b>	<b>62.3 ± 5.78</b>	5.12 ± 0.11	54.3 ± 4.88	2.65 ± 0.16	42.2 ± 3.94	3.21 ± 0.22	43.4 ± 3.46	<b>7.48 ± 0.67</b>	<b>74.5 ± 6.12</b>

<sup>a</sup>Symbol notation, see Table 2.1

<sup>b</sup>MNS mean number of shoots per explant

<sup>c</sup>MSI multiple shoot induction

<sup>d</sup>N CaCl<sub>2</sub> content in the nutrient medium 220 mg/l

<sup>e</sup>2N CaCl<sub>2</sub> content in the nutrient medium 440 mg/l

<sup>f</sup>The largest values of micropropagation efficiency are in bold type

### 2.3.3 Rooting of Microclones and Their Transfer to Soil

The percentage of microclones that formed roots on the MS/2 medium, supplemented with 0.1 mg/l NAA, was 44–85 and varied for different species. This value was highest for *G. asclepiadea* and the lowest for *G. punctata* (Pozhzyzhivska mountain), *G. lutea* (Rivna mountain valley), and *G. acaulis* (Turkul mountain) (Table 2.5). Microclones of the two latter genotypes rooted better on the medium with increased calcium. Use of 440 mg/l  $\text{CaCl}_2$  increased rooting of G.l.Riv 5.7 fold and G.ac.T 7.1 fold (Table 2.5).

The number of viable, morphologically normal plants that to transfer to soil was greatest among *G. asclepiadea* from both localities (40.2 % and 45.2 %) and *G. pneumonanthe* (the village of Vyhoda; 41.7 %) but the least in *G. verna* (8.6 %) (Table 2.5).

The comparison of own and reference data showed the advantage of using explants from axenic plants. Long-time sterilization of explants of wild plants is inefficient (the percentage of infection in *G. lutea* reached 89) (Feijoo and Iglesias 1998) which may lead to a decrease of their viability that is undesirable during rare plant multiplication. Both in research by the present authors and in work by other

**Table 2.5** Results of rooting microclones and adaptation of plants to growth in soil

Sample	Rooting shoots in vitro	Adaption of plants to growth in soil		
	No. of shoots	Rooting (%)	No. of plants	Adaptation (%)
G.l.R <sup>a</sup>	98	59.2 ± 4.6	52	17.3 ± 1.2
G.l.Tr	116	63.8 ± 5.9	66	16.7 ± 1.5
G.l.P	129	67.4 ± 6.1	81	28.4 ± 1.3
G.l.Riv	36	13.9 ± 5.8	4	— <sup>b</sup>
	102 <sup>c</sup>	79.4 ± 4 <sup>c</sup>	69	34.2 ± 4.68
G.ac.Reb	60	53.3 ± 4.8	28	21.4 ± 1.2
G.ac.T	20	10 ± 6.7	2	—
	63 <sup>c</sup>	71.4 ± 5.7 <sup>c</sup>	37	28.6 ± 6.5
G.p.Br	51	54.9 ± 5.1	26	19.2 ± 1.5
G.p.Tr	62	51.6 ± 4.4	25	16 ± 1.3
G.p.P	47	44.6 ± 4.1	20	15 ± 1.4
G.asc.P	98	85.7 ± 7.3	72	40.2 ± 3.7
G.asc.M	128	81.2 ± 7.4	93	45.4 ± 4.1
G.cr.K	109	76.1 ± 6.1	75	37.3 ± 3.7
G.cr.M	98	71.4 ± 7.2	63	33.3 ± 2.7
G.pn.K	113	63.7 ± 4.9	68	36.8 ± 3.2
G.pn.V	115	68.7 ± 6	72	41.7 ± 3.7
G.v.G	133	57.1 ± 4.2	58	8.6 ± 0.6

<sup>a</sup>Symbol notation, see Table 2.1

<sup>b</sup>Plants died

<sup>c</sup>Results obtained on MS/2 medium with double  $\text{CaCl}_2$  amount

authors positive results on gentians micropropagation were obtained predominantly on MS-based medium (Momcilović et al. 1997; Hosokawa et al. 1996, 1998; Feijoo and Iglesias 1998; Butiuc-Keul et al. 2005). However, Momcilović et al. (1997) described a greater multiplication efficiency in vitro for *G. acaulis* on MS medium with macronutrients of Woody Plant Medium (Lloyd and McCown 1980). A better results were obtained for that species due to an increase of calcium. The decisive role in gentian microclone formation relates to PGRs (Momcilović et al. 1997; Feijoo and Iglesias 1998). Efficient multiplication of the seven species of gentians investigated was obtained by introducing into the medium only of BAP and Kin as cytokinins. Although it has been reported that successful establishment of *Gentiana* cultures requires a medium containing both a cytokinin and an auxin, for example BA or TDZ and indole-3-acetic acid (IAA) (Momcilović et al. 1997; Feijoo and Iglesias 1998). The auxin NAA was used for rooting regenerated shoots. In the work by Momcilović et al. (1997), 35–70 % of shoots of *G. cruciata*, *G. purpurea*, and *G. acaulis* rooted spontaneously, with the exception of *G. lutea*, in which adventitious roots were induced by applying NAA. The optimum results of *G. lutea* micropropagation efficiency were the same as those mentioned in the literature (mean number of shoots reached 6.5 per explant); for *G. cruciata* and *G. acaulis*, the efficiency was more than times that for other investigations (Momcilović et al. 1997).

Conditions were chosen for micropropagation of plants representing 16 genotypes of 7 gentian species. Optimal for multiplication was MS/2 medium, supplemented with 0.05–0.5 mg/l BAP and 0.1–0.2 mg/l Kin. For 2 genotypes, *G. lutea* (Rivna mountain valley) and *G. acaulis* (Turkul mountain), better microclone formation was observed when  $\text{CaCl}_2$  was doubled in the medium. On optimum medium, the percentage of microclonal cuttings constituted 62–92 %, and the number of adventitious shoots formed per cutting was 2.6–7.5. The number of rooted microclones was 45–86 %. The best adaptation to ex vitro conditions was by *G. asclepiadea* and *G. pneumonanthe*. *G. verna* with the best capacity for multiplication showed the worst adaptation to soil.

## 2.4 Callus Induction and Proliferation

The results shown in Tables 2.6, 2.7, 2.8, 2.9, and 2.10 prove that the ability for callus formation among the gentian investigated was significantly different. On optimal media, the frequency of callus formation for most species reached 100 %, with the exception of *G. verna* and *G. acaulis* for which the this value was 88 and 70 %, respectively. For *G. pneumonanthe* and *G. acaulis*, the efficiency of callus production depended on the genotype with variation between 18–20 % and 10 %, respectively (Tables 2.7 and 2.9).

*G. asclepiadea* stood out among the species investigated. Its distinguishing feature was that formed on the MS and MS/2 medium callus stopped growing after

**Table 2.6** Frequency of callus induction (%) from root, shoot, and leaf explant of four *G. lutea* genotypes on different media

Growth regulators, mg/l	Medium	G.I.R <sup>a</sup>						G.I.Tr						G.I.Riv						G.I.P					
		Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf
BAP	NAA																								
	0.4	MS	6.3 ± 0.4	16.8 ± 1.4	4.5 ± 0.2	17.4 ± 1.2	4.8 ± 0.3	7.8 ± 0.4	17.4 ± 1.2	4.8 ± 0.3	6.3 ± 0.4	14.3 ± 1.1	4.3 ± 0.2	8.3 ± 0.6	19.2 ± 1.7	5.4 ± 0.4									
		MS/2	28.2 ± 2.2	18.7 ± 1.5	8.7 ± 0.7	19.2 ± 1.1	9.4 ± 0.7	26.4 ± 1.2	19.2 ± 1.1	9.4 ± 0.7	8.5 ± 0.7	15.8 ± 1.3	6.5 ± 0.4	28.3 ± 2.3	19.5 ± 1.4	8.9 ± 0.7									
BAP		2.4-D																							
0.1	0.5	MS	<b>100<sup>b</sup></b>	78.6 ± 4.8	38.9 ± 2.9	83.2 ± 6.3	49.8 ± 3.8	95.6 ± 7.2	83.2 ± 6.3	49.8 ± 3.8	35.8 ± 2.9	18.3 ± 1.5	32.4 ± 2.3	<b>100</b>	93.3 ± 6.2	73.2 ± 4.5									
		MS/2	<b>100</b>	<b>91.3 ± 7.4</b>	<b>56.4 ± 4.4</b>	<b>96.4 ± 7.9</b>	<b>64.2 ± 4.5</b>	<b>100</b>	<b>96.4 ± 7.9</b>	<b>64.2 ± 4.5</b>	48.6 ± 3.6	32.9 ± 2.7	<b>40.8 ± 2.9</b>	<b>100</b>	<b>100</b>	<b>80 ± 5.6</b>									
0.1	1	MS	42.5 ± 2.9	32.8 ± 1.7	12.8 ± 1.1	34.6 ± 2.8	14.4 ± 1.1	39.2 ± 3.1	34.6 ± 2.8	14.4 ± 1.1	46.2 ± 3.9	20.5 ± 1.4	10.2 ± 0.9	40.4 ± 3.1	32.3 ± 2.2	7.4 ± 0.4									
		MS/2	84.3 ± 5.1	54.6 ± 3.2	25.9 ± 1.3	58.4 ± 4.5	27.3 ± 2.3	68.7 ± 5.5	58.4 ± 4.5	27.3 ± 2.3	<b>92.4 ± 7.5</b>	<b>80.6 ± 4.2</b>	29.4 ± 2.2	70.5 ± 5.4	60.4 ± 4.8	39.8 ± 2.9									

<sup>a</sup>Symbol notation, see Table 2.1

<sup>b</sup>The largest values of callus formation are in bold type

**Table 2.7** Dependence of *G. acaulis* and *G. verna* callus induction frequency (%) on explant type and nutrient medium composition

Growth regulators (mg/l)		Medium	G.ac.T <sup>a</sup>				G.ac.Reb				G.v.G			
BAP	NAA		Root	Shoot	Leaf	Root	Root	Shoot	Leaf	Shoot	Root	Root	Shoot	Leaf
2	0.4	MS	8.5 ± 0.5	4.2 ± 0.2	3.8 ± 0.2	9.3 ± 0.6	9.3 ± 0.6	3.8 ± 0.2	4.1 ± 0.2	6.2 ± 0.5	6.2 ± 0.5	8.4 ± 0.6	3.4 ± 0.2	
		MS/2	11.7 ± 1	9.4 ± 0.6	6.3 ± 0.4	12.3 ± 1	12.3 ± 1	10.2 ± 0.8	8.1 ± 0.6	9.2 ± 0.7	9.2 ± 0.7	13.4 ± 1.1	5.3 ± 0.4	
BAP	2,4-D													
0.1	0.5	MS	22.8 ± 1.8	16.7 ± 1.2	11.4 ± 1.1	58.6 ± 4.7	58.6 ± 4.7	48.8 ± 3.9	46.4 ± 3.1	67.3 ± 5.4	67.3 ± 5.4	76.4 ± 6.1	22.1 ± 1.4	
		MS/2	30.5 ± 2.5	23.4 ± 2.1	19.5 ± 1.1	<b>69.5<sup>b</sup></b> ± <b>5.5</b>	<b>69.5<sup>b</sup></b> ± <b>5.5</b>	<b>50.1 ± 4.1</b>	<b>48.3 ± 3.2</b>	<b>71.4 ± 4.3</b>	<b>71.4 ± 4.3</b>	<b>88.2 ± 5.9</b>	<b>24.8 ± 2.1</b>	
0.1	1	MS	29.4 ± 2.2	18.2 ± 1.4	15.3 ± 1.3	31.2 ± 2.4	31.2 ± 2.4	20.4 ± 1.7	16.5 ± 1.2	65.2 ± 5.5	65.2 ± 5.5	71.8 ± 6.1	21.2 ± 1.8	
		MS/2	<b>49.6 ± 4.1</b>	<b>34.5 ± 3.2</b>	<b>28.4 ± 2.4</b>	54.3 ± 3.4	54.3 ± 3.4	40.2 ± 3.2	35.4 ± 3.1	67.3 ± 5.4	67.3 ± 5.4	76.4 ± 6.1	22.1 ± 1.4	

<sup>a</sup>Symbol notation, see Table 2.1  
<sup>b</sup>The largest values of callus formation are in bold type

**Table 2.8** Frequency of callus induction (%) from different explant of three *G. punctata* genotypes

Growth regulators (mg/l)	Medium	G.p.P <sup>a</sup>				G.p.Br				G.p.Tr			
		Root	Shoot	Leaf		Root	Shoot	Leaf		Root	Shoot	Leaf	
BAP	NAA												
2	0.4	MS	21.3 ± 1.6	18.4 ± 1.6	9.3 ± 0.6	23.8 ± 1.1	15.8 ± 1.1	8.2 ± 0.5		23.8 ± 2.1	20.5 ± 1.7	15.2 ± 1.3	
		MS/2	25.2 ± 2.2	20.4 ± 1.7	8.2 ± 0.6	26.4 ± 2.2	18.3 ± 1.5	7.9 ± 0.5		30.2 ± 2.2	24.3 ± 2.1	12.4 ± 1.1	
BAP	2,4-D												
0.1	0.5	MS	95.4 ± 7.6	80.8 ± 6.2	24.6 ± 1.9	86.4 ± 6.7	82.4 ± 5.6	22.3 ± 1.9		92.3 ± 7.1	89.6 ± 6.3	32.2 ± 2.9	
		MS/2	<b>100<sup>b</sup></b>	<b>100</b>	<b>35.7 ± 2.9</b>	<b>98.3 ± 8.5</b>	<b>90.4 ± 7.5</b>	<b>32.8 ± 2.7</b>		<b>100</b>	<b>100</b>	<b>40.3 ± 3.4</b>	
0.1	1	MS	14.3 ± 1.2	9.3 ± 0.8	6.4 ± 0.5	13.5 ± 1.1	8.2 ± 0.6	6.2 ± 0.4		15.5 ± 1.1	11.2 ± 0.8	8.9 ± 0.7	
		MS/2	69.6 ± 5.1	60.2 ± 5.5	19.3 ± 1.6	63.8 ± 5.5	55.9 ± 3.7	17.8 ± 1.2		78.3 ± 6.2	62.4 ± 5.3	20.8 ± 1.8	

<sup>a</sup>Symbol notation, see Table 2.1  
<sup>b</sup>The largest values of callus formation are in bold type



**Table 2.9** Dependence of *G. cruciata* and *G. pneumonanthe* callus induction frequency (%) on explants type and nutrient medium composition

Growth regulators (mg/l)		Medium	G.cr.Kr <sup>a</sup>				G.cr.Med				G.pn.K				G.pn.V			
BAP	NAA		Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf	Root	Shoot	Leaf	Root
2	0.4	MS	7.2 ± 0.4	10.3 ± 0.8	5.6 ± 0.4	9.4 ± 0.7	15.9 ± 1.4	7.9 ± 0.6	9.2 ± 0.7	3.1 ± 0.2	6.3 ± 0.4	10.2 ± 0.7	6.4 ± 0.4	8.9 ± 0.6				
		MS/2	9.4 ± 0.7	16.4 ± 1.2	7.8 ± 0.5	21.6 ± 1.8	19.6 ± 1.5	10.5 ± 0.9	12.6 ± 1.1	11.3 ± 0.8	8.8 ± 0.6	14.5 ± 1.1	16.3 ± 1.1	10.8 ± 0.9				
BAP	2.4-D																	
0.1	0.5	MS	65.6 ± 5.3	48.6 ± 3.6	39.8 ± 3.1	24.6 ± 2.2	61.4 ± 4.8	32.2 ± 2.9	69.8 ± 4.3	68.6 ± 3.9	42.8 ± 3.1	86.4 ± 7.6	78.3 ± 6.5	55.6 ± 4.6				
		MS/2	78.4 ± 6.2	54.3 ± 4.6	48.8 ± 3.3	38.9 ± 3.1	49.8 ± 4.2	38.2 ± 2.8	<b>80.4<sup>b</sup></b> ± <b>6.8</b>	<b>74.6 ± 5.4</b>	<b>54.3 ± 4.9</b>	<b>98.3 ± 8.1</b>	<b>84.7 ± 7.2</b>	<b>60.2 ± 5.1</b>				
0.1	1	MS	94.8 ± 8.3	66.3 ± 5.6	52.4 ± 4.9	79.8 ± 6.5	<b>80.8 ± 7.2</b>	42.9 ± 3.9	51.2 ± 4.4	63.4 ± 3.2	38.3 ± 2.4	60.4 ± 4.2	71.2 ± 6.1	44.3 ± 3.1				
		MS/2	<b>100</b>	<b>85.4 ± 6.5</b>	<b>79.8 ± 5.2</b>	<b>89.4 ± 6.9</b>	67.7 ± 5.5	<b>55.8 ± 4.9</b>	46.3 ± 3.2	48.7 ± 4.1	33.7 ± 2.8	70.3 ± 5.9	54.1 ± 4.1	40.2 ± 3.2				

<sup>a</sup>Symbol notation, see Table 2.1

<sup>b</sup>The largest values of callus formation efficiency are in bold type

**Table 2.10** Frequency of callus induction (%) from root, shoot, and leaf explants of two *G. asclepiadea* genotypes on different nutrient media

Growth regulators (mg/l)		Medium	G.asc.P <sup>a</sup>			G.asc.M		
BAP	NAA		Root	Shoot	Leaf	Root	Shoot	Leaf
2	0.4	MS	28.9 ± 2.6	18.3 ± 1.2	6.2 ± 0.4	31.3 ± 2.6	9.2 ± 2.9	11.7 ± 1.2
		MS/2	30.8 ± 2.7	11.2 ± 0.9	8.5 ± 0.5	29.5 ± 2.6	7.6 ± 0.7	15.3 ± 1.4
BAP	2,4-D							
0.1	0.5	MS	92.3 ± 8.6	68.6 ± 5.9	18.4 ± 1.4	86.7 ± 6.9	67.8 ± 5.3	37.1 ± 2.5
		MS/2	<b>100<sup>b</sup></b>	<b>75 ± 6.3</b>	<b>20.3 ± 1.2</b>	<b>94.1 ± 2.4</b>	<b>78.5 ± 4.1</b>	<b>37.9 ± 2.9</b>
0.1	1	MS	60.2 ± 4.3	42.3 ± 3.5	11.3 ± 1.1	57.3 ± 5.0	40.8 ± 3.9	19.7 ± 1.8
		MS/2	75.1 ± 6.9	50.4 ± 3.5	16.2 ± 1.1	77.4 ± 4.2	48.6 ± 4.8	34.4 ± 2.8
		B <sub>5</sub>	61 ± 4.9	51 ± 2.7	43.7 ± 3.1	82.4 ± 7.1	62.5 ± 4.6	83.3 ± 6.9
0.2	1	B <sub>5</sub>	68.5 ± 4.2	<b>83.5 ± 6.6</b>	<b>74 ± 5.5</b>	83.3 ± 6.8	50 ± 3.5	58.3 ± 4.1
Kin	2,4-D							
0.2	1	B <sub>5</sub>	<b>74 ± 4.6</b>	78 ± 4.5	63 ± 5.1	<b>94.7 ± 7.7</b>	<b>91.4 ± 8.5</b>	<b>95.1 ± 8.5</b>

<sup>a</sup>Symbol notation, see Table 2.1

<sup>b</sup>The largest values of callus formation are in bold type

some time, become brown, and then developed necrosis (Strashniuk et al. 2004). However, callus initiation on B<sub>5</sub> medium was generally more with stable growth.

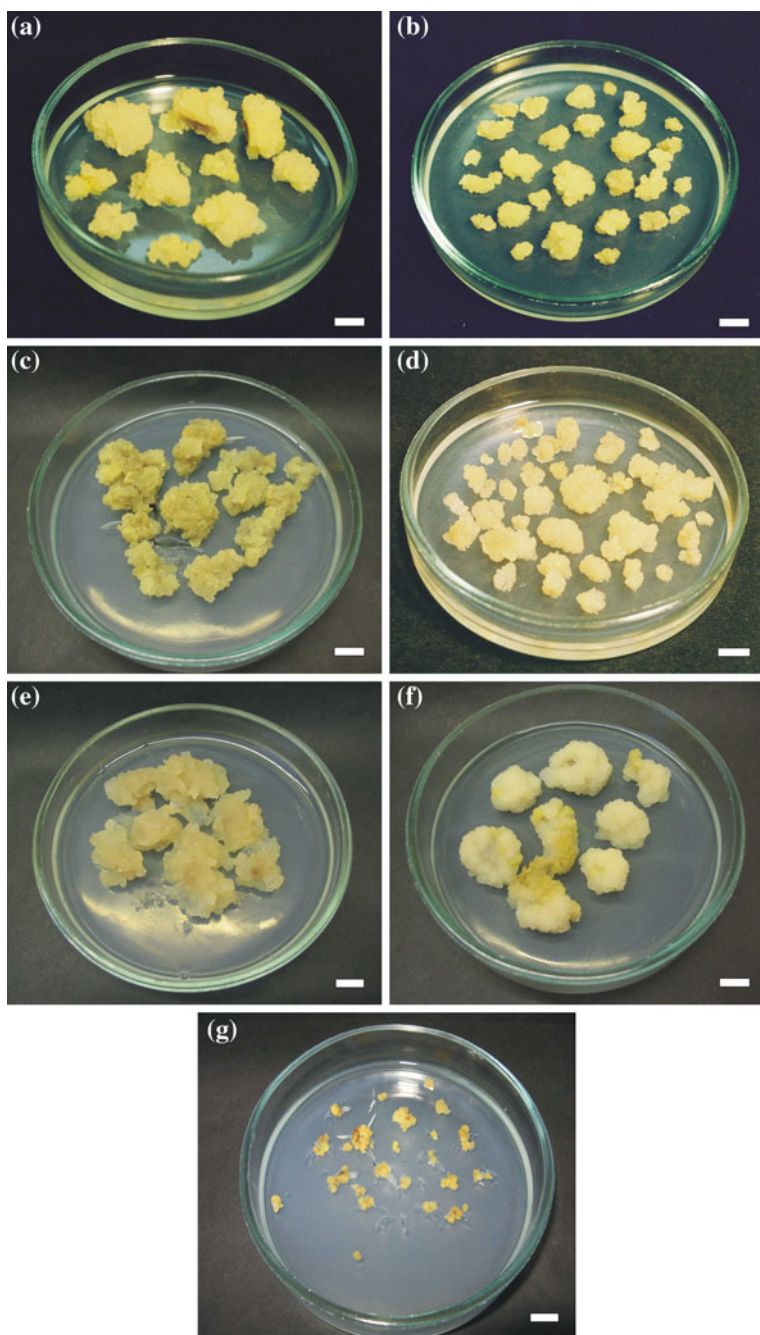
A comparison of callus formation on MS-based medium with full and half concentration of salts showed considerable (1.5–4 times) advantage of the latter (MS/2) for all samples except genotype G.cr.Med. The growth regulators had the most effect on callus formation. BAP and NAA together induced insignificant CI; CI was 3–31 %. The callus grew slowly and with necrosis after a subculture. The use of BAP (0.1 mg/l) and 2,4-D (0.5, 1 mg/l) stimulated formation of callus of a light yellow color, of soft consistency that grew rapidly. Callus formation was best on medium supplemented with 0.1 mg/l BAP and 0.5 mg/l 2,4-D. However, for some genotypes such as G.l.Riv, G.ac.T, G.cr.Kr, and G.cr.Med, callus formation was more intensive in the presence of 1 mg/l 2,4-D (Tables 2.6, 2.7 and 2.9). On B<sub>5</sub> medium *G. asclepiadea* apart from BAP but at presence of Kin (0.2 mg/l) favored high CI values (>90 %) for the G.asc.M genotype. For the other genotype G.asc.P, a combination of 0.2 mg/l Kin and 1 mg/l 2,4-D on B<sub>5</sub> medium was better for root explants but for stem and leaf explants most callus formation was with 0.2 mg/l BAP instead of Kin (Table 2.10).

For different explant types, the optimal medium composition was usually the same. The exceptions were several genotypes, the roots, stems, and leaf explants of which formed callus best of all on the media with salts content (G.cr.Med), or growth regulators (G.l.Riv, G.asc.P). Root explants exhibited the best callus formation, whereas leaf explants showed the least callus formation. The exceptions were *G. asclepiadea* samples, and CI values for leaf explants approximated to these of roots and stems ones.

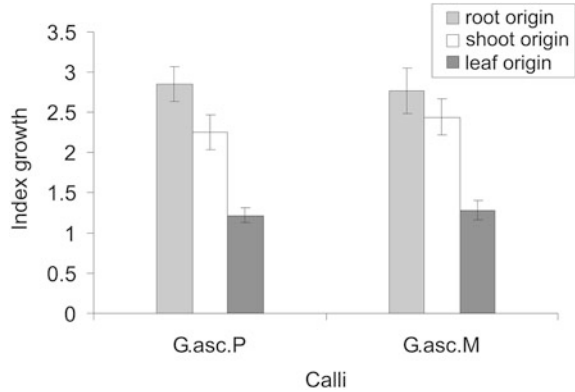
MS/2 medium was optimal for callus proliferation of most species except for *G. verna* where MS medium has shown better results (Fig. 2.4). Both callus formation and proliferation in *G. asclepiadea* were more efficient on the B<sub>5</sub> medium (Fig. 2.5).

There were no essential interspecific differences in growth of cultured tissues (Fig. 2.6). Somewhat, larger GI values were characteristic of *G. asclepiadea*, *G. cruciata*, *G. pneumonanthe*, and *G. punctata* calli. The combination of BAP and 2,4-D stimulated growth of all the cultures. The genotypes of same species proliferated efficiently with equal concentrations of growth regulators. Only in the case of *G. lutea* were the best results for three genotypes obtained with 0.1 mg/l BAP, whereas for G.l.Riv it was necessary to increase this cytokinin twofold (Fig. 2.7). In general, for half of the genotypes, investigated concentrations of 0.1 mg/l BAP and 0.5 mg/l 2,4-D were optimal. Some calli required a double concentration of cytokinin (0.2 mg/l BAP) for efficient proliferation. For efficient callus proliferation in *G. asclepiadea*, it was essential to introduce into the nutrient medium twice as much cytokinin and auxin (0.2 mg/l BAP and 1 mg/l 2,4-D).

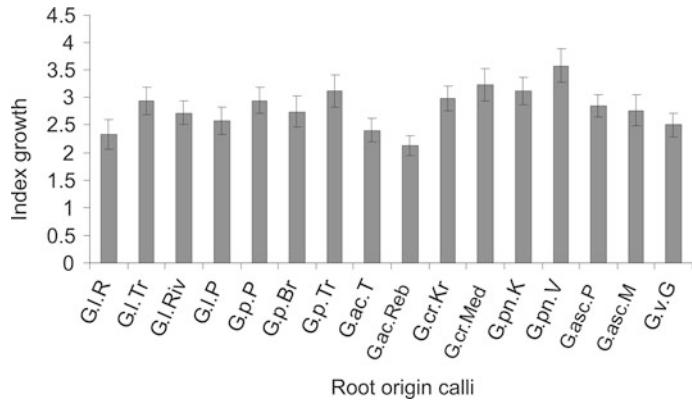
Initial explants had a substantial influence on proliferation efficiency. Growth indices on optimal nutrient media were the highest for calli from root explants (2.1–3.6) (Fig. 2.6), somewhat lower from stems (1.8–2.9) while the lowest was from leaf explants (0.6–1.3). Callus of *G. acaulis* and *G. verna* of leaf origin was incapable of proliferation. For other species investigated, the growth of leaf callus within 2–3 (*G. lutea*) and 5–7 (*G. punctata*, *G. cruciata*, and *G. pneumonanthe*)



**Fig. 2.4** Callus cultures of root origin. **a** *G. lutea*, **b** *G. punctata*, **c** *G. acaulis*, **d** *G. asclepiadea*, **e** *G. pneumonanthe*, **f** *G. cruciata*, **g** *G. verna*. Bars = 1 cm

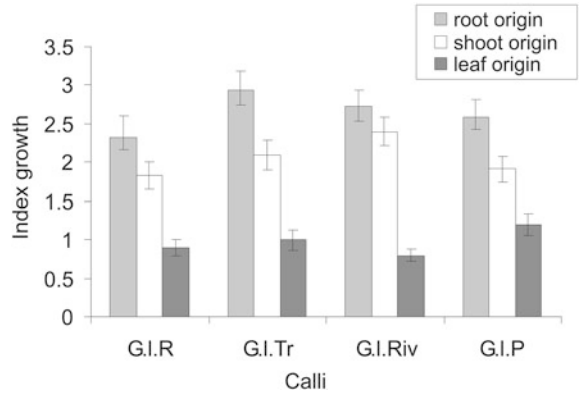


**Fig. 2.5** Growth index of tissues from different explants of *G. asclepiadea* on optimum nutrient medium



**Fig. 2.6** Growth index of root origin calli of gentians on optimum medium

**Fig. 2.7** Growth index of different tissues of *G. lutea* on optimal nutrient media: with 0.1 mg/l BAP + 0.5 mg/l 2,4-D for G.I.R, G.I.Tr, G.I.P; and 0.2 mg/l BAP + 0.5 mg/l 2,4-D for G.I.Riv



subcultures gradually slowed down with darkening and necrosis. Only callus of *G. asclepiadea* leaf explants was capable of continuous growth.

The literature indicates certain complexity of obtaining and proliferating callus of the species investigated. According to the authors (Demkiv 1993; Skrzypczak et al. 1993), it is connected with phenolic compounds in these plants; for rapid callus growth, usually 2,4-D and BAP or Kin were used (Wesołowska et al. 1985; Skrzypczak et al. 1993).

In our study we obtained callus from 7 gentian species and three types of explants (root, stem and leaf). BAP and 2,4-D growth regulators were necessary both for callus induction and growth. MS/2 supplemented with 0.1–0.2 mg/l BAP and 0.3–1 mg/l 2,4-D was found to be optimal for callus induction on all types of explants in six species (*G. punctata*, *G. cruciata*, *G. pneumonanthe*, *G. lutea*, *G. acaulis*, *G. verna*). In the case of *G. asclepiadea* callus formation required B<sub>5</sub> medium supplemented with double concentration of both cytokinin and auxin (0.2 mg/l BAP and 1 mg/l 2,4-D). The efficiency of callus formation from root and stem explants resulted in 100 % for some species. Leaf explants had significantly less callus initiation capacity in most species. The culture media optimal for callus proliferation were MS/2 for *G. punctata*, *G. cruciata*, *G. pneumonanthe*, *G. lutea*, *G. acaulis*; MS for *G. verna*; B<sub>5</sub> for *G. asclepiadea*.

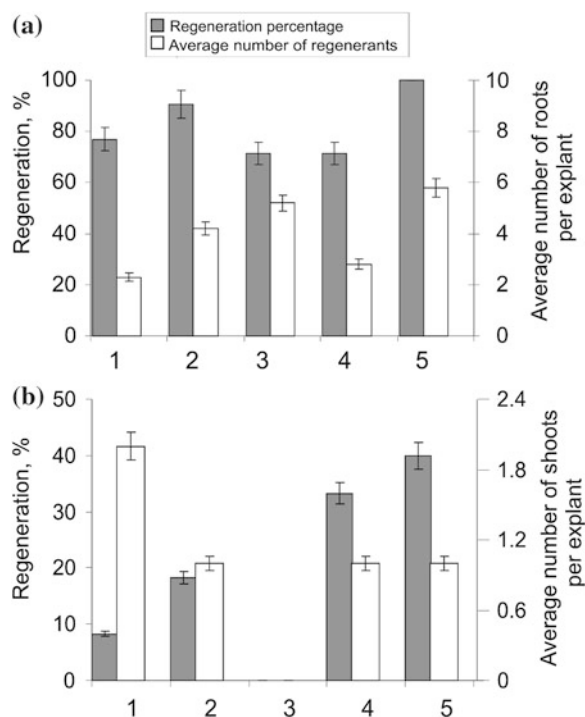
## 2.5 Direct Organogenesis of *G. lutea*

While choosing conditions for regeneration, the efficiency of *G. lutea* organogenesis depended on the concentration of TDZ and NAA in the medium, and the original genotype as well as the type of explant (Konvalyuk et al. 2010).

### 2.5.1 The Influence of Exogenous Growth Regulators on Regeneration Efficiency

On the medium with 1 mg/l TDZ and 0.01 mg/l NAA, there occurred rhizogenesis, the percentage of which (see Eq. 2.5) from root explants of the Troyaska mountain plants was 76.9 %, MNR (see Eq. 2.6) 2.3 roots/explant, shoot formation from stem explants RP was 8.3 %, and MNR 2 shoots/explant (Fig. 2.8a, b). MS medium with 5 mg/l TDZ (NAA 0.01 mg/l) increased root RP and the value of MNR to 90.6 %, with 4.2 roots/explant from Troyaska mountain plants and to 92.3 %, with 2.8 roots/explant from Lemska (Figs. 2.8a and 2.9a). On this medium, shoot RP from explants of G.I.Tr plants increased more than twice and constituted 18.2 %, while MNR decreased 2 times to 1 shoot/explant. Shoot regeneration from G.I.L plans was not observed.

**Fig. 2.8** Rhizogenesis on root (a) and shoot formation on stem (b) explants of *G. lutea* from the Troyaska mountain. MS medium contained: (1) 1 mg/l TDZ and 0.01 mg/l NAA; (2) 5 mg/l TDZ and 0.01 mg/l NAA; (3) 10 mg/l TDZ and 0.01 mg/l NAA; (4) 20 mg/l TDZ and 0.01 mg/l NAA; (5) 5 mg/l TDZ and 1 mg/l NAA

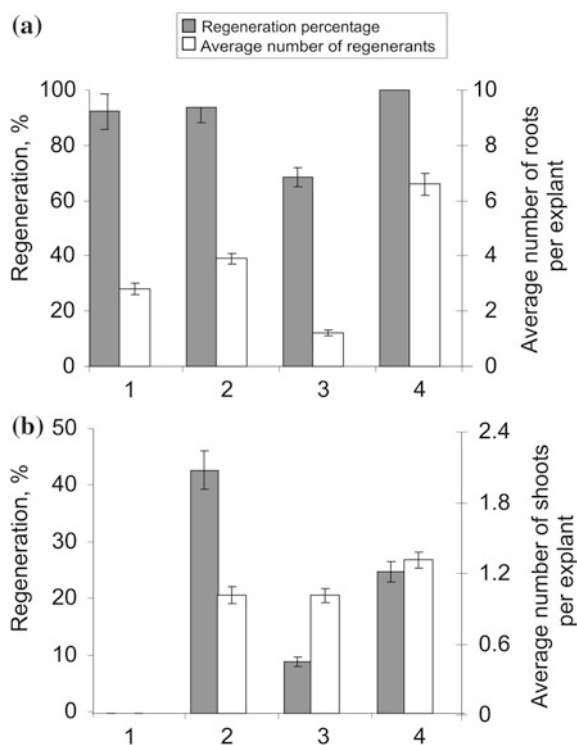


TDZ concentration increased from 10 to 20 mg/l with 0.01 mg/l NAA led to decreased rhizogenesis with the MNR index decreased 1.9–4.1 times in all the investigated variants (Figs. 2.8a, 2.9, and 2.10a). The percentage of shoot formation from stem explants taken from plants of Lemska and Pozhyzhevskia localities decreased 2.3–4.7 times but MNR indices were unchanged (Figs. 2.9b and 2.10b). On medium with 20 mg/l TDZ and 0.01 mg/l NAA shoot formation percentage was the greatest in the case of stem explants of plants from the Troyaska mountain (33.3 %) with an MNR value of 1 shoot/explant (Fig. 2.8b). With 5 mg/l TDZ and 1 mg/l NAA, shoot RP from plants of this locality was somewhat higher (40 %), although the MNR value was the same.

The increase of NAA concentration from 0.01 to 1 mg/l in the nutrient medium facilitated the increase of both rhizogenesis efficiency indices: RP to 71.4–100 % and MNR to 1.7–6.6 roots/explant (Figs. 2.8a, 2.9 and 2.10a).

For most samples, investigated rhizogenesis efficiency was the highest when 5–10 mg/l TDZ and 1 mg/l NAA were used; the regeneration percentage from root explants was 100 %, MNR index was within 4.1–6.6 roots/explant (Figs. 2.8a and 2.9a). On the medium supplemented with 10 mg/l TDZ and 100 times decreased concentration of NAA (0.01 mg/l), RP from root explants (plants of Pozhyzhevskia locality) was not high (57.1 %), unlike the MNR number that reached 9.5 roots/explant and was the highest among the samples investigated (Fig. 2.10a).

**Fig. 2.9** Rhizogenesis on root (a) and shoot formation on stem (b) explants of *G. lutea* from the Lemska mountain valley. MS medium contained: (1) 5 mg/l TDZ and 0.01 mg/l NAA; (2) 10 mg/l TDZ and 0.01 mg/l NAA; (3) 20 mg/l TDZ and 1 mg/l NAA; (4) 10 mg/l TDZ and 1 mg/l NAA



Nutrient medium with 10 mg/l TDZ and 0.01 mg/l NAA favoured efficient shoot regeneration from stem explants of Lemska (RP—42.9 %, MNR—1 shoot/explant) and Pozhizhevskaya (RP—40 %, MNR—1 shoot/explant) plants (Figs. 2.9b and 2.10b). Regeneration capacity of stem explants of Troyaska plants was the highest on medium with 5 mg/l TDZ and 1 mg/l NAA (RP—40 %, MNR—1 shoot/explant) (Fig. 2.8b).

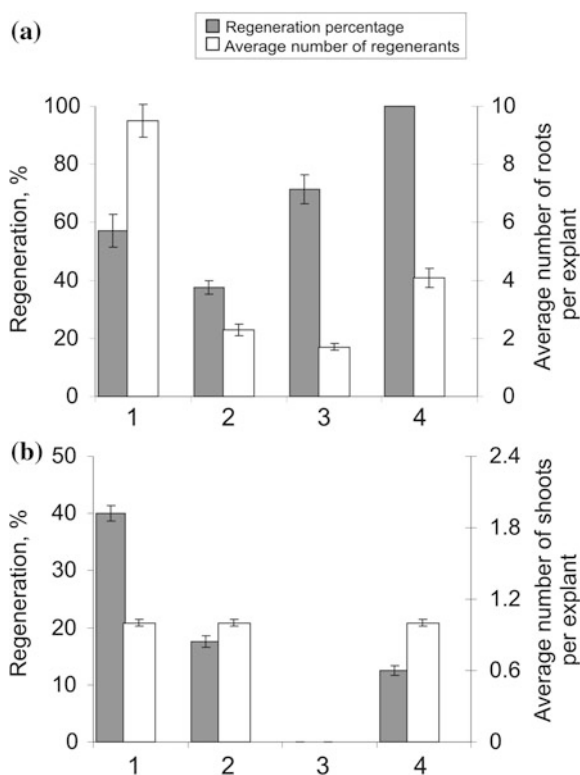
### 2.5.2 Regeneration Dependence on Genotype

Organogenesis efficiency indices for plants from four localities on nutrient medium with 10 mg/l TDZ and 0.01 mg/l NAA differed considerably (Fig. 2.11).

Rhizogenesis was optimum on root explants from plants of Pozhizhevskaya mountain (RP—57.1 %, MNR—9.5 roots/explant, RE (see Eq. 2.7)—5.4 regenerants/explant) (Fig. 2.11a). A comparatively high RE index (3.7 regenerants/explant) was obtained in regeneration from Troyaska and Lemska plants (RP and MNR constituted 71.4 % and 5.2 roots/explant and 93.8 % and 3.9 roots/explant,



**Fig. 2.10** Rhizogenesis on root (a) and shoot formation on stem (b) explants of *G. lutea* from the Pozhyshevskaya mountain. MS medium contained: (1) 10 mg/l TDZ and 0.01 mg/l NAA; (2) 20 mg/l TDZ and 0.01 mg/l NAA; (3) 5 mg/l TDZ and 1 mg/l NAA; (4) 10 mg/l TDZ and 1 mg/l NAA

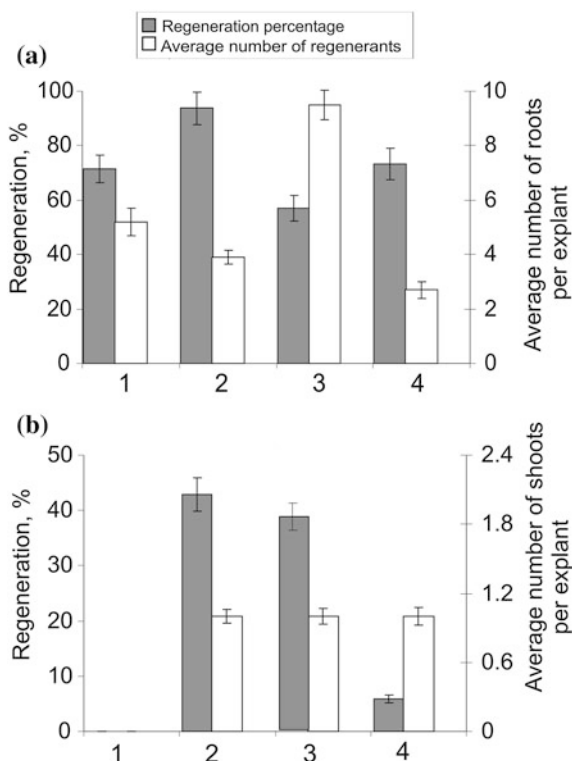


respectively). In the sample investigated, relatively low capacity for rhizogenesis was observed among explants from Rohneska mountain valley plants, the regeneration efficiency of which was 2.7 regenerants/explant, while RP was 73.3 % and MNR 3.7 roots/explant (Fig. 2.11a; Table 2.11).

Shoot formation was most efficient in the case of stem explants from Lemska (RP—42.9 %, MNR—1 shoot/explant) and Pozhyshevskaya (RP—40 %, MNR—1 shoot/explant) plants (Fig. 2.11b). For Rohneska mountain valley plants, RP was approximately 7 times lower in comparison with 2 previously mentioned localities. Shoot regeneration from explants of Troyaska mountain plants was not observed on this variant medium.

In general, explants from plants of different genotypes had different regeneration capacities under the same conditions of cultivation. At the same time, the efficiency of morphogenic potential for different genotypes was determined by concentration and ratio of growth regulators in nutrient medium.

**Fig. 2.11** Rhizogenesis on root (a) and shoot formation on stem (b) explants of *G. lutea* from different localities: (1) Troyaska; (2) Lemska; (3) Pozhyzhevsk; (4) Rohneska. MS medium with 10 mg/l TDZ and 0.01 mg/l NAA



### 2.5.3 Evaluation of the Regeneration Efficiency from Different Explants

The capacity to regeneration in vitro was shown by stem and root explants from plants of all genotypes. Leaf explants on the different media darkened and become necrotic. In most cases, shoots formed on stem explants, sometimes both rhizogenesis and callus formation or only callus formation occurred. Callus formed on stem explants from Pozhyzhevsk (10 mg/l TDZ and 0.01 mg/l NAA) and Lemska (5 mg/l TDZ and 0.01 mg/l NAA) plants. Simultaneous formation of callus and roots was observed on one stem explant from Lemska mountain valley plants (10 mg/l TDZ and 0.01 mg/l NAA).

Rhizogenesis was observed mainly on root explants, and only in one case (G.I.L.) with 5 mg/l TDZ and 0.01 mg/l NAA, was there simultaneous callus and root formation.

The mean value of root regeneration efficiency from root explants from four localities investigated constituted 3.1 regenerants/explant; shoots from stem explants—0.17 regenerants/explant (Table 2.11). Other indices of regeneration

**Table 2.11** Direct regeneration from root, stem, and leaf explants of *G. lutea*

Locality	No. of plant-donors	No. of planted explants	No. of explants with regenerants	RP <sup>a</sup> , %	MNR <sup>b</sup>	RE <sup>c</sup>
<i>Root explants</i>						
Troyaska	10	128	103	82.1 ± 3.4	4.05	3.41
Lemska	6	104	88	88.6 ± 3.1	3.64	3.43
Pozhyzhevskaya	5	48	31	66.5 ± 6.8	4.4	2.9
Rohneska	2	15	11	73.3 ± 11.4	3.72	2.73
Total for four localities	23	295	233	75.1 ± 2.5	3.95	3.1
<i>Stem explants</i>						
Troyaska	10	40	8	20 ± 6.3	1	0.216
Lemska	6	54	8	19.2 ± 5.4	0.83	0.213
Pozhyzhevskaya	5	32	6	17.5 ± 6.7	0.75	0.18
Rohneska	2	17	1	5.9 ± 5.7	1	0.06
Total for four localities	23	143	23	15.6 ± 3.0	0.9	0.17
<i>Leaf explants</i>						
Troyaska	10	11	0	— <sup>d</sup>	—	—
Lemska	6	5	0	—	—	—
Pozhyzhevskaya	5	5	0	—	—	—
Rohneska	2	2	0	—	—	—
Total for four localities	23	23	0	—	—	—

<sup>a</sup>RP regeneration percentage<sup>b</sup>MNR mean number of regenerants per explant with regenerants<sup>c</sup>RE regeneration efficiency<sup>d</sup>Regeneration did not occur

capacity (RP and MNR) during shoot formation were also considerably lower when compared to rhizogenesis.

Differences in regeneration capacity were found for *G. lutea* explants. Leaf explants on the variant-tested media were not capable of regeneration. Shoots formation and rhizogenesis occurred on stem explants but rhizogenesis on root explants only. On both types of explants, alongside morphogenesis some callus formation was also observed.

The direct regeneration of *G. lutea* has been established in vitro. The reaction of plants from four localities to culture conditions (PGRs content) differed significantly (Figs. 2.8, 2.9, 2.10, and 2.11). There were also differences both in regeneration percentage and in the number of regenerants per explant.

Regeneration indices were practically the same for plants from Lemska and Troyaska localities and were the highest in the samples (Table 2.11). The value of rhizogenesis efficiency for plants from Pozhyzhevskaya and Rohneska localities was lower (2.9 and 2.7 regenerants/explant, respectively). The efficiency of shoot

regeneration from stem explants of Rohneska mountain valley plants was the lowest in the samples; 3–3.6 times lower in comparison with the plants from other localities (Table 2.11).

It is known that the plant genotype influences on regeneration indices. Various genotypes under the same conditions display different morphogenetic reaction (Kushnir and Sarnats'ka 2005; Kunakh 2005).

Morphogenic potential in *G. lutea* also depended on the type of explant. It was peculiar for plants from all the genotypes investigated that only roots formed from root explants, but stem explants produced predominantly shoots. Only in case of *G. l.L* three roots did form on one stem explant.

Root explants were characterized by much bigger regeneration capacity than those from stems. The mean value of RE from root explants was 18.2 times higher than from stem explants (Table 2.11). However, organogenesis from stem and leaf explants on medium identical of composition was less efficient or did not take place proving the necessity to optimize further the culture conditions for shoot regeneration.

Regenerants were not obtained from leaf explants of all *G. lutea* plants on any medium. Attempts by of other researches to initiate regeneration of adventitious shoots from leaf explants of the species mentioned as well as *G. punctata* were also unsuccessful (Skrzypczak et al. 1993). These results illustrated the dependence of *G. lutea* regeneration capacity on culture conditions, especially on PGRs. However, there are some species, namely *G. pneumonanthe*, *G. kurroo*, and *G. macrophylla* which showed somatic embryos regeneration potential on leaf blade (Bach and Pawłowska 2003; Fiuk and Rybczyński 2008; Chen et al. 2009).

Previous research was aimed at the conditions selection for *G. lutea* organogenesis with the use of various combinations of the cytokinins BAP and Kin, and the auxins NAA and 2,4-D. However, only initial stages of root and shoot regeneration were induced without viable regenerants. According to the literature sources, TDZ is preferable in comparison with other cytokinins for induction of organogenesis in many plants (Ellis et al. 1991), including gentians (Hosokawa et al. 1996; Bach and Pawłowska 2003). While choosing conditions for regeneration of 9 commercial *Gentiana* species, TDZ was a more efficient cytokinin than 4-CPPU (*N*-(2-chloro-4-pyridyl)-*N'*-phenylurea), BA and zeatin, and NAA more efficient as an auxin than IAA or 2,4-D (Hosokawa et al. 1996).

These results prove that the use of TDZ and NAA in MS medium induced regeneration of *G. lutea* shoots and roots. Regeneration efficiency depended not only on the concentration of growth regulators but also on their ratio. Adding to the medium 10 mg/l TDZ and 1 mg/l NAA was optimal for rhizogenesis from root explants of *G. lutea* plants from the Lemska mountain valley. In other cases, 10 mg/l TDZ and 0.01 mg/l NAA were optimal for obtaining in vitro regenerants from stem and root explants of Pozhyshevskaya mountain plants, and 5 mg/l TDZ and 1 mg/l NAA for stem and root explants from Troyaska mountain plants.

These PGRs, TDZ and NAA, were used for regeneration of adventitious shoots from leaf and stem (5–10 mg/l TDZ and 0.1 mg/l NAA) and from root (10 mg/l

TDZ and 1 mg/l NAA) explants of commercial cultivars of *Gentiana* (Hosokawa et al. 1996).

Thus, *G. lutea* has the capacity to form adventitious shoots and roots by means of direct regeneration. Realization in vitro of morphogenic potential depends on the genotype, explant type, and culture conditions. Organogenesis efficiency from root explants exceeded considerably the efficiency from stem explants. On leaf explants regeneration did not occurred. More information concerning *G. lutea* morphogenic potential is presented in Chap. 6 of this Volume.

## 2.6 Plant Regeneration from Tissue Culture

The optimal combination for organogenesis was MS medium supplemented with 10 mg/l TDZ and 1 mg/l NAA. In two subcultures, regeneration occurred from callus of *G. pneumonanthe* and *G. cruciata* on light condition and at the end of the 3–4th subcultures (*G. pneumonanthe* and *G. cruciata*) the regeneration of roots and shoots was observed. In other samples (except of *G. verna*) on the same medium some callus turned green. In *G. lutea*, the formation of regeneration loci occurred after 4–5 months. Organogenic parts remained unchanged for the next 3–4 subcultures. In *G. acaulis* (Rebra mountain) the formation of regeneration areas was detected after 7 subcultures. The next (eighth) subculture caused rhizogenesis. In callus of species obtained Turkul mountain all inocula turned green in the 7–8th subcultures on the same medium but regeneration loci did not form. *G. verna* tissues remained unchanged for 2 subcultures, the 3–5th ones resulted in callus darkening, and at the 6th subculture areas gray-green in color began to form.

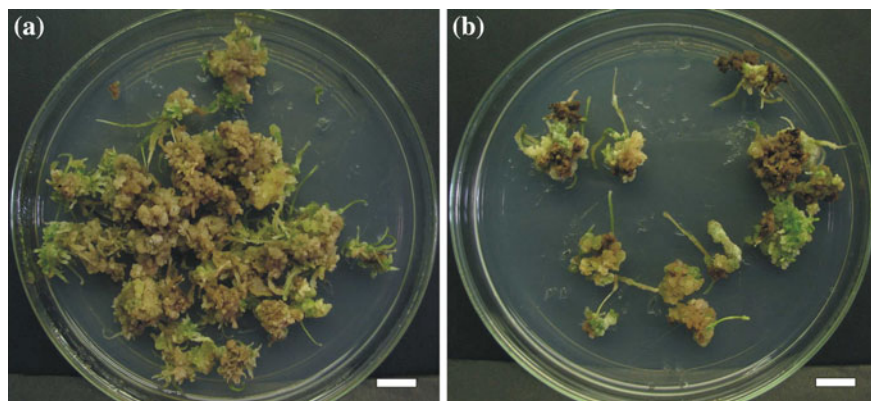
On media with other concentrations of TDZ and NAA, as well as with BAP and NAA, callus only proliferated, and there was no obvious regeneration (with all concentrations of BAP and NAA). In addition to proliferation, some small areas first turned green and then remained unchanged pending further cultivation (with 1 or 5 mg/l TDZ); there was also tissue darkening and necrosis (20 mg/l TDZ).

In *G. pneumonanthe* calli, shoot and root regeneration was observed, but from *G. cruciata* and *G. acaulis* tissues only roots regenerated. Calli of *G. pneumonanthe* of both geographic locations showed highest PR of roots and MNRhiz. These indices were the lowest for *G. acaulis* (Table 2.12).

Organogenesis depended on the original genotype. In particular, the number of roots per explant (under the same rhizogenesis percentage) in *G. pneumonanthe* cultures obtained from the Vyhoda village plant exceeded by 2.3 times this value in callus from plant of Koriukivka forestry. Shoot regeneration percentage as well as root regeneration in the cultures was practically the same, but the number of shoots per explant in callus from the Koriukivka locality plant was twice as many (Table 2.12). *G. cruciata* callus obtained from Krenychi village samples showed 1.5 times higher RP of roots and 1.7 higher MNRhiz than callus from “Medobory” nature reserve samples (Table 2.12).

**Table 2.12** Rhizogenesis and shoot formation efficiency in tissue cultures of some *Gentiana* species

Genotype	Subculture	Efficiency of rhizogenesis		Efficiency of shoot formation	
		RP <sup>a</sup> of roots, %	MNRhiz <sup>b</sup> , roots/explant	RP of shoots, %	MNS <sup>c</sup> , shoots/explant
G.pn.V <sup>d</sup>	9-th	100	21.7 ± 1.7	17.4 ± 2.1	0.2 ± 0.02
	19-th	81.8 ± 6.5	6.6 ± 0.5	9.1 ± 0.8	0.5 ± 0.04
G.pn.K	9-th	100	9.3 ± 0.8	16.7 ± 1.4	0.4 ± 0.04
G.cr.M	13-th	25 ± 1.9	0.38 ± 0.04	— <sup>e</sup>	—
G.cr.Kr	14-th	36.4 ± 3.7	0.63 ± 0.05	—	—
G.ac.Reb	17-th	16.7 ± 1.7	0.17 ± 0.02	—	—

<sup>a</sup> RP regeneration percentage<sup>b</sup> MNRhiz mean number of roots per explant with roots<sup>c</sup> MNS mean number of shoots per explant with shoots<sup>d</sup> Symbolic notation see in Table 2.1<sup>e</sup> Regeneration did not take place**Fig. 2.12** Organogenesis from *G. pneumonanthe* morphogenic callus at 9th (a) and 19th (b) subcultures on MS medium with 10 mg/l TDZ and 1 mg/l NAA. Bars = 1 cm

The capacity for organogenesis of *G. pneumonanthe* calli depended on the term of their growth. Thus, with an increase in culture period (the village of Vyhoda) from 9 to 19 subcultures, the general number of regenerants (roots and shoots) per explant decreases threefold (Fig. 2.12). Rhizogenesis efficiency for this species was 10 (in one case—100 times) higher than shoot organogenesis (Table 2.12).

Regenerants were obtained only from morphogenic tissues of *G. pneumonanthe* plant from the Koriukivka forestry. Rooting of regenerants took place on MS/2 medium with 0.2 mg/l BAP and 1 mg/l GA<sub>3</sub>. On other media, regenerants become necrotic within 3–5 months. Further transplanting of regenerated plants was on medium with twice decreased concentrations of PGRs. The regenerants were characterized by intense growth and well-developed root systems.

Among the species investigated, the best capacity for regeneration was found in *G. pneumonanthe* culture. MS medium supplemented with 10 mg/l TDZ and 1 mg/l NAA was optimal for organogenesis. This depended on both the genotype and the term of callus growth. *G. pneumonanthe* rhizogenesis efficiency was 10 times (in one case 100) higher than shoot organogenesis.

## 2.7 Fast-Growing Isolated Root Culture

At the first stage, the optimal for all explants was medium with 0.1 mg/l BAP (*G. lutea*, *G. punctata*, *G. cruciata*) or Kin (*G. acaulis*, *G. asclepiadea*, *G. pneumonanthe*) (Konvalyuk et al. 2011). The concentration of NAA varied from 0.3 to 2 mg/l depending on the origin of inocula (Table 2.13). Thus, for instance for *G. punctata*, *G. asclepiadea*, *G. cruciata*, and *G. pneumonanthe* samples from different localities, the same concentrations of NAA were necessary, namely 0.5, 0.3, 2, and 0.5 mg/l, respectively. However, *G. lutea* for efficient isolated root formation, concentrations of NAA varied from 0.5 to 2 mg/l, depending on plant genotype. *G. asclepiadea* stood out among the species, for its culture, unlike for other gentians, B<sub>5</sub> medium was used. Besides, in the case of *G. acaulis*, intensive growth of isolated roots was stimulated MS/2 medium with double CaCl<sub>2</sub> concentration (440 mg/l). The GI and yield (fresh weight) of *G. acaulis* isolated root culture were 47 times higher than those on the medium with standard concentration of CaCl<sub>2</sub> (220 mg/l).

Analysis of results showed that gentian root inocula were characterized by a different capacity to form isolated roots (Fig. 2.13). Thus, the mean number of side rootlets per inoculum after the first stage of culture was the biggest in *G. lutea* (81–101) and *G. acaulis* (53–95) while the worst capacity to form side rootlets was in *G. punctata* (35–63) and *G. asclepiadea* (35–44). The species differed by the extent of side rootlet growth. After the second culture stage the mean root size was longest in *G. lutea* (19.2–30.8 mm) and the shortest in *G. punctata* and *G. asclepiadea* (17.4–21.3 mm and 18.2–18.6 mm, correspondently) (Table 2.13).

The greatest value of GI was for *G. lutea* and *G. cruciata*. In some cases, various genotypes of the same species differed considerably by the extent of side rootlet growth. Most distinctions referred to *G. pneumonanthe*, as well as to *G. lutea* from Troyaska mountain when compared to those from other habitats.

Thus, the use of two-stage culture enables formation of a considerable number of side rootlets (in some cases >100 per inoculum), essential increase of side rootlet length (up to 30 mm), and their rapid growth (GI reached 926.5) (Table 2.13).

Among the isolated root cultures obtained, the highest biomass was in *G. lutea* plants (Troyaska mountain), with 225 g per 1 l of medium that equals to the root mass of 10- to 12-year-old plant in nature (Strashniuk et al. 2005). The GI of this culture was 926.5 and corresponded to values obtained by other authors after their use of transformation by *Agrobacterium rhizogenes*. For example, the GI of nine clones of transformed *G. lutea* roots were 150.8–1473.2 (Menković et al. 2000).

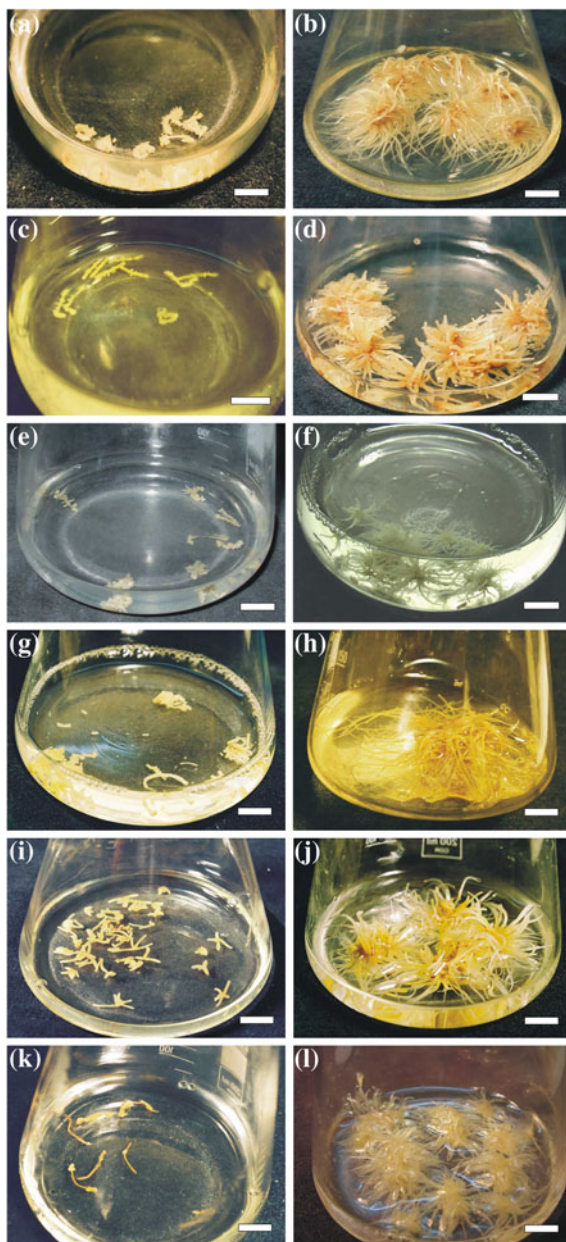
**Table 2.13** Some growth parameters for isolated root culture of gentians at optimal concentrations of growth regulators

Genotype	Concentration of plant growth regulators in the medium (l culture stage) <sup>a</sup>	Mean number of side rootlets per inoculum	Mean length of side rootlets, mm		Grows index by fresh weight	Yield of biomass after 4–6 weeks of cultivation per 1 l of medium, g
			I culture stage	II culture stage		
G.I.Riv <sup>b</sup>	0.1 mg/l BAP + 0.5 mg/l NAA	93 ± 7.9	4.5 ± 0.3	24.1 ± 1.5	385 ± 31.7	84.7 ± 5.6
G.I.R	0.1 mg/l BAP + 2 mg/l NAA	84 ± 6.7	4.2 ± 0.2	22.4 ± 1.9	312.8 ± 25.2	81.52 ± 5.4
G.I.P	0.1 mg/l BAP + 1 mg/l NAA	101 ± 9.3	5.5 ± 0.3	27.5 ± 2.1	428 ± 36.4	85.62 ± 6.5
G.I.Tr		98 ± 8.5	6.3 ± 0.4	30.8 ± 2.9	926.5 ± 84.4	225.5 ± 16.7
G.I.L		81 ± 6.8	3.8 ± 0.2	19.2 ± 1.1	298.5 ± 22.5	71.64 ± 4.9
G.p.P	0.1 mg/l BAP + 0.5 mg/l NAA	63 ± 4.1	4.1 ± 0.38	21.3 ± 1.7	308.3 ± 28.3	67.82 ± 4.9
G.p.Br		35 ± 2.3	3.2 ± 0.2	17.4 ± 1.5	192.8 ± 17.4	42.4 ± 3.1
G.p.Tr		42 ± 3.4	3.8 ± 0.2	18.8 ± 1.4	203.4 ± 16.3	44.6 ± 2.9
G.ac.T	0.1 mg/l Kin + 0.5 mg/l NAA	53 ± 3.8	1.5 ± 0.3	7.06 ± 0.8	6.95 ± 0.5	1.52 ± 0.1
		95 ± 7.3 <sup>c</sup>	4.2 ± 0.5 <sup>c</sup>	23.06 ± 1.6 <sup>c</sup>	324 ± 25.7 <sup>c</sup>	71.28 ± 5.7 <sup>c</sup>
G.asc.P	0.1 mg/l Kin	35 ± 2.9	3.4 ± 0.2	18.2 ± 1.1	216.4 ± 17.4	51.94 ± 3.3
G.asc.M	+0.3 mg/l NAA	44 ± 3.4	3.8 ± 0.2	18.6 ± 1.2	250.2 ± 18.6	55.04 ± 4.1
G.cr.Kr	0.1 mg/l BAP	83 ± 7.1	5.1 ± 0.4	28.21 ± 2.2	654.2 ± 52.3	143.92 ± 12.8
G.cr.Med	+2 mg/l NAA	79 ± 5.4	4.8 ± 0.4	25.18 ± 2.2	505.3 ± 32.3	121.28 ± 9.9
G.pn.K	0.1 mg/l Kin	89 ± 6.4	4.8 ± 0.3	30.14 ± 2.5	686.4 ± 45.3	151.02 ± 13.2
G.pn.V	+0.5 mg/l NAA	76 ± 5.8	4.3 ± 0.3	20.14 ± 1.5	289.3 ± 19.8	69.44 ± 4.7

<sup>a</sup>Half strength MS medium (except G.asc.P and G.asc.M cultured on B<sub>5</sub> medium)<sup>b</sup>Symbol notation, see Table 2.1<sup>c</sup>The results are obtained on MS/2 medium with double CaCl<sub>2</sub> concentration



**Fig. 2.13** Isolated root cultures at the I (a, c, e, g, i, k) and II (b, d, f, h, j, l) stages of cultivation; (a, b) *G. lutea*, (c, d) *G. punctata*, (e, f) *G. asclepiadea*, (g, h) *G. pneumonanthe*, (i, j) *G. cruciata*, (k, l) *G. acaulis*. Bars = 1 cm



We revealed that GI of isolated root cultures was significantly higher (60–300 times) than the GI of proliferating calli of the same species (see Sect. 2.4).

Fast-growing root cultures of six *Gentiana* species were obtained and the characteristics of their growth were investigated. Gentians have a capacity to form

root cultures that depended on genotype as well as medium composition. The cultures differed considerably by their growth parameters as in the quantity and length of side rootlets, GI by fresh weight and biomass production.

## 2.8 Conclusions

Tissue and organs cultures were investigated of *Gentiana* species from the Ukrainian flora. The capacity to form morphogenic or non-morphogenic cultures and their growth depended on the original genotype, the type of explants and composition of the medium, as well as content and ratio of PGRs. Selected conditions for micropropagation resulted in a both, high percentage of cuttings with microclones and the number of adventitious shoots formed. In some cases, callus formation efficiency from root and stem explants was 100 %. The regeneration efficiency of adventitious shoots and roots through the direct organogenesis in vitro from *G. lutea* stem and root explants reached 3.4 regenerants per explant. Among the species investigated, the best capacity for indirect regeneration was found in *G. pneumonanthe*. The extent of organogenesis is depended on the period of callus growth. Fast-growing root cultures were characterized by a high growth rate and considerable biomass yield. Selection of the composition of media and conditions of growth provide a high efficiency of propagation and considerable biomass of gentian cultures in vitro.

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