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Genotype and plant growth regulator-dependent response of somatic embryogenesis from G*entiana* spp. leaf explants

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Abstract *Gentiana kurroo* (Royle), *Gentiana cruciata* (L.), *Gentiana tibetica* (King. ex Hook. f.), *Gentiana lutea* (L.), and *Gentiana pannonica* (Scop.) leaves derived from axenic shoot culture were used as explants. For culture initiation, leaves from the first and second whorls from the apical dome were dissected and cultured on Murashige and Skoog (MS) basal medium supplemented with three different auxins: 2,4-dichlorophenoxyacetic acid, 1-naphthaleneacetic acid (NAA), or 3,6-dichloro-*o*-anisic acid (dicamba) in concentrations of 0.5, 1.0, or 2.0 mg/l; and five different cytokinins: zeatin, 6-furfurylamonopurine (kinetin), *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea (TDZ), *N*-(2-chloro-4-pyridyl)*N*′-phenylurea, or 6-benzylaminopurine (BAP). The cytokinin concentrations used were dependent on the type of cytokinin and varied between 0.25 and 3.0 mg/l. After 2 mo. of culture, the morphogenic response of explants was assessed. Frequency of embryogenesis was the highest for *G. kurroo* (54.7%) and dependent on plant growth hormones (PGRs). This gentian was the only species showing morphogenic capabilities on media supplemented with all applied combinations of PGRs, while none of the 189 induction media permutations stimulated somatic embryogenesis from *G. lutea* explants. *G. tibetica* and *G. cruciata* both produced an average of 6.6 somatic embryos per explant, while *G. pannonica* and *G. kurroo* regenerated at 15.7 and 14.2 somatic embryos per explant, respectively. Optimum regeneration was achieved in the presence of NAA combined with BAP or TDZ. This auxin also stimulated abundant rhizogenesis. Somatic embryos were also regenerated from adventitious roots of *G. kurroo*, *G. cruciata*, and *G. pannonica*. Somatic embryos converted into plantlets on half strength MS medium.

Keywords *Gentiana* sp. · Leaf explants · Somatic embryogenesis · Adventitious roots

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Introduction

Plants in the genus *Gentiana* play an important role in ethnobotany, pharmacology, and horticulture, with many species legally protected by law throughout the world (Clarke et al. 1998; Köhlein 1991). Due to their commercial value, several *Gentiana* species are of interest to plant biotechnology. Some are characterized by high production of secondary metabolites, while others are valued in horticulture production because of the beauty of their flowers.

Micropropagation offers the potential to produce millions of clonal individuals though tissue culture via induction of morphogenesis from various plant tissues or organs. This method is commonly employed for the mass propagation of crop species and ornamental plants and for the conservation of genetic resources (Hempel 1989; Fay 1992). To date, 14 *Gentiana* species including *Gentiana acaulis*, *Gentiana cruciata*, *Gentiana lutea* (Momčilović et al. 1997), *Gentiana cerina*, *Gentiana corymbifera* (Morgan et al. 1997), *Gentiana kurroo* (Sharma et al. 1993) *Gentiana triflora*, and *Gentiana triflora x scabra* (Hosokawa et al. 1996) have been studied for their capacity to respond to *in vitro* multiplication. In all cases, however, only organogenesis has been described and with only a few shoots recovered per primary explant.

Somatic embryogenesis is a highly efficient process for plant multiplication. We previously reported studies describing the potential of both seedling explants and suspension cultures to produce somatic embryos in *Gentiana tibetica*, *G. cruciata*, *Gentiana pannonica*, and *G. kurroo* (Mikuła and Rybczyński 2001; Mikuła et al. 2002, 2005; Fiuk et al. 2003). Those papers studied induction, maintenance, and preservation of embryogenic competence of explants originating from zygotic embryos and seedling organs.

The developing plant provides numerous sources of tissue, characterized by different morphogenic potentials. The aim of this paper was to assess the embryogenic potential of leaf explants from five gentian species that are the subject of biotechnological interest. The *Gentiana* genus consists of species that vary widely in leaf size and shape (Köhlein 1991). The leaf is expected to be the most convenient explant since it has a high morphogenic potential, is easy to collect, can produce green mesophyll protoplasts, and is a potential target for transformation. In the present study, leaf explants were analyzed for the induction of somatic embryogenesis, somatic embryo production, and germling uniformity in the presence of varying concentrations of plant growth regulators.

Materials and Methods

Plant material. Five gentian species were investigated: *G. cruciata* L., *G. kurroo* Royle, *G. lutea* L., *G. pannonica*

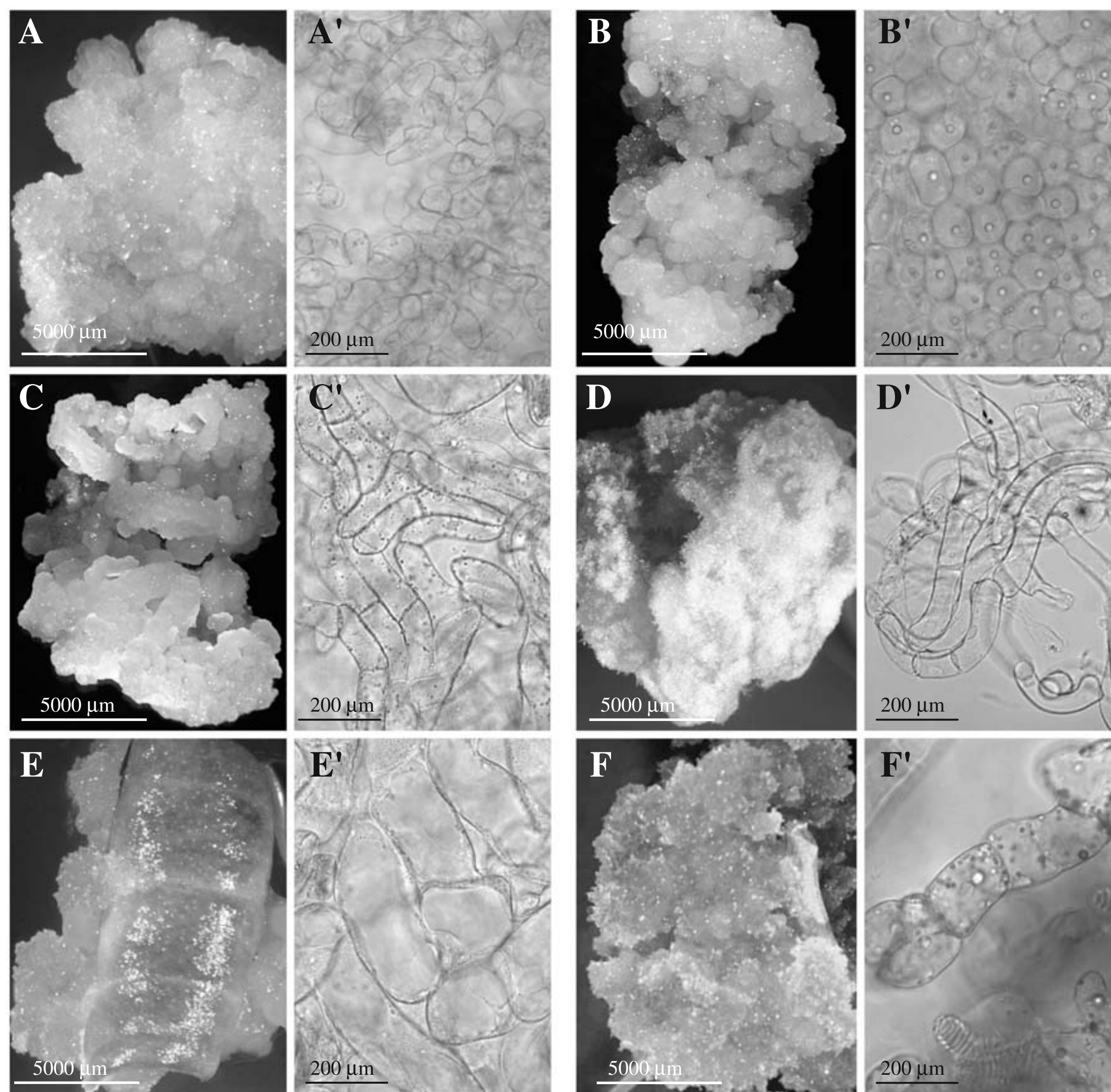

Figure 1. Types of callus tissue observed developing from leaf explants of *Gentiana* species. *A* and *A′* Fine embryogenic callus—*G. kurroo* (MS + 1.0 mg/l Dic + 1.0 mg/l TDZ). *B* and *B′* Nodule embryogenic callus—*G. cruciata* (MS + 1.0 mg/l 2,4-D + 0.5 mg/l Kin). *C* and *C′* Filiform non-embryogenic callus—*G. cruciata* (MS + 2.0 mg/l 2,4-D). *D* and *D′* White non-embryogenic callus—*G. tibetica* (MS + 0.5 mg/l NAA + 1.0 mg/l TDZ). *E* and *E′* Hydrated non-embryogenic callus—*G. kurroo* (MS + 1.0 mg/l 2,4-D + 0.25 mg/l Zeat). *F* and *F′* Green non-embryogenic callus—*G. pannonica* (MS + 2.0 mg/l NAA).

Scop., and *G. tibetica* King ex Hook. f. Seeds were purchased from the Polish Academy of Sciences' Botanical Garden—Center for Biological Diversity Conservation (*G. cruciata* and *G. pannonica*), Grugapark, Essen in Germany (*G. kurroo*), Gottingen in Germany (*G. lutea*), and the Rotterdam Zoo and Botanical Garden (*G. tibetica*) and stored at 4°C. The seeds were sterilized in 20% *v/v* commercial bleach (Domestos) for 15 min followed by three washes in sterilized water. Seeds were germinated on Murashige and Skoog (MS; Murashige and Skoog 1962) basal medium solidified with 0.8% *w/v* agar and supplemented with 0.5 mg/l gibberelic acid (GA_3) and 30 g/l sucrose. Media was adjusted to pH 5.8 and then autoclaved at 121°C for 18 min. Seeds germinated under conditions of 22±1°C and 16 h of daily light from cool-white fluorescent lamps at 50 μEm^{-2} s^{1}. Seedlings with two leaves were transferred onto the same basal medium containing 2.0 mg/l benzylaminopurine (BAP), 0.2 mg/l naphthalene acetic acid (NAA), and 30 g/l sucrose for multiplication. Shoots 2–3 cm in length were excised from mother plants and transferred onto medium without plant growth hormones (PGRs) to develop axenic shoot cultures. Shoot cultures were maintained at the same temperature and light conditions described above. Experimental leaf explants were excised from the first and second leaf whorls below the apical dome. Whole leaves, from petiole to apex, were cut into 0.5-cm cross-sections and placed onto the induction medium with the abaxial side in contact with the medium.

Culture conditions. MS basal medium supplemented with 30 g/l sucrose was utilized in all experiments to induce somatic embryogenesis. Three concentrations (0.5, 1.0 and 2.0 mg/l) of NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), and 3,6-dichloro-*o*-anisic acid (Dic) in combination with 6-furfurylamonopurine (Kin), zeatin (Zeat), *N*-(2-chloro-4-pyridyl)*N*′-phenylurea (CPPU); (0.0, 0.25, 0.5, 1.0, 2.0 mg/l), and *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea (TDZ) and BAP (0.0, 0.5, 1.0, 2.0, 3.0 mg/l) were used for experiments. Five Petri dishes for each combination were established. Explants from two or three leaves, depending on their size, were placed onto one plate. Cultures were maintained for 2 mo. in darkness at 22±1°C without subculture. Somatic embryos produced from explant-derived tissues at the cotyledonary stage were transferred directly onto half strength MS medium supplemented with 30 g/l sucrose and 0.8% (*w/v*) agar and were cultured at 22±1°C, 16-h light (100 μEm^{-2} s^{-1}), and 8-h dark conditions for conversion to plantlets.

Statistical evaluation. After 2 mo. of culture, frequency of rooting and somatic embryogenesis (percentage of explants forming roots and somatic embryos) and mean number of somatic embryos per explants (with standard deviation) were scored.

Determination of DNA content. In order to evaluate the DNA content of the regenerated plantlets, a Partec (Münster, Germany) CCA flow cytometer with argon laser was used. Experiments were performed according to Thiem and Śliwińska (2003). Young leaves were chopped with a sharp razor blade in Petri dish with 1,000 µl nucleus-isolation buffer (0.1 M Tris, 2.5 mM $MgCl_2 \times 6\ H_2O$, 85 mM NaCl, 0.1% Triton X-100; pH 7.0) supplemented by propidium iodide (50 mg/ml) and RNase (50 µg/ml). After cutting, the suspension was passed through a 50-µm mesh nylon filter, and about 10,000 nuclei were analyzed. Histograms were analyzed using a DPAC V.2.2 computer program. Leaves of green pea 'Set' (2C=9.11 pg DNA) served as internal standard. Plants obtained from seeds, which had not been though embryogenic culture, were used as the control. The 2C value of somatic embryo-derived regenerants was compared to that of control plants. At least 30 regenerants per species were checked.

Table 1. Embryogenesic potential of leaf explants of 5 *Gentiana* species

Species	PGR combinations used														
	2,4-D					NAA					DIC				
	Kin	Zeat	BAP	TDZ	CPPU	Kin	Zeat	BAP	TDZ	CPPU	Kin	Zeat	BAP	TDZ	CPPU
G. kurroo	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G. cruciata	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−
G. pannonica	−	−	−	−	−	+	+	+	+	+	−	−	−	−	+
G. tibetica	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+
G. lutea	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Leaf explants were cultured for 2 mo. in the dark at 22±1°C on MS basal medium supplemented with three concentrations: 0.5, 1.0, and 2.0 mg/l of NAA (1-naphthaleneacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), and Dic (3,6-dichloro-*o*-anisic acid) in combination with 0.0, 0.25, 0.5, 1.0, 2.0 mg/l Kin (6-furfurylamonopurine), Zeat (zeatin), CPPU (*N*-(2-chloro-4-pyridyl)*N*′-phenylurea) or 0.0, 0.5, 1.0, 2.0, 3.0 mg/l BAP (6-benzylaminopurine) and TDZ (*N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea).

Results

Callus induction. The first indications of explant tissue dedifferentiation appeared in the area of the vascular bundles. A few days later, callus tissue was seen to have spread over the edge of the excision surface and then the abaxial side of the leaf explant. Build-up of vascular bundles in the petiole caused callus to appear at once on all surfaces and to grow rapidly. In controls cultured on hormone-free MS medium, explants remained green and

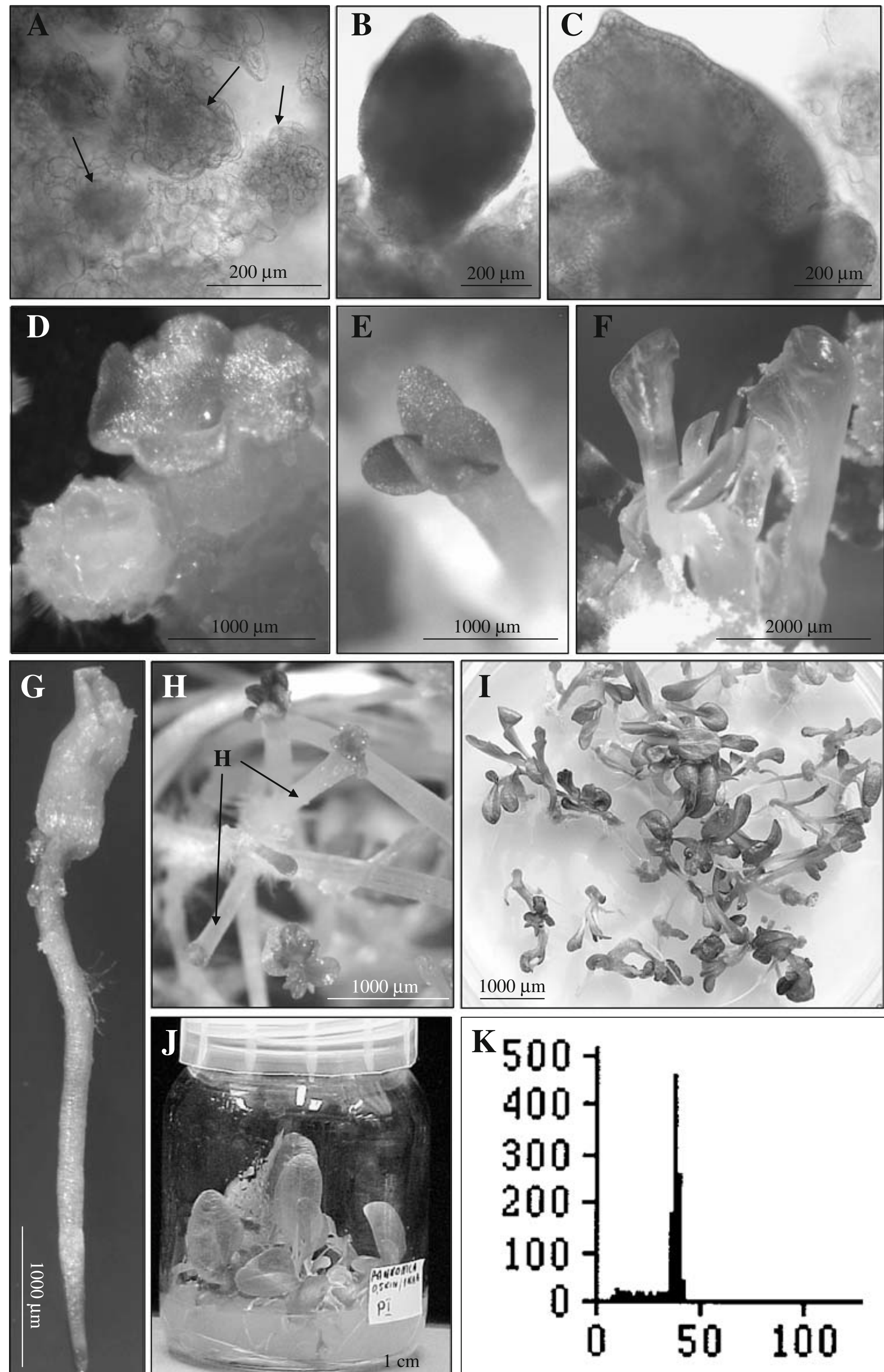

Figure 2. Somatic embryogenesis on leaf explants of gentians. (*A*) Embryogenic centers in the callus tissue of *G. kurroo* on MS medium supplemented with 1.0 mg/l 2,4-D and 2.0 mg/l CPPU. (*B*) Somatic embryo of *G. kurroo* in heart stage. (*C*) Somatic embryo of *G. kurroo* in torpedo stage. (*D*) Somatic embryo of *G. kurroo* with grown cotyledons on MS medium supplemented with 1.0 mg/l Dic and 0.5 mg/l Kin. (*E*) Somatic embryo of *G. tibetica* with separated cotyledons on MS medium supplemented with 0.5 mg/l NAA and 0.25 mg/l Kin. (*F*) Vitrified somatic embryos of *G. tibetica* on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l TDZ. (*G*) Somatic embryo of *G. kurroo* with very long rootlets on MS medium supplemented with 0.5 mg/l NAA and 3.0 mg/l TDZ. (*H*) Somatic embryos of *G. pannonica* with long hypocotyls on MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l Kin. (*I*) Conversion of somatic embryos *G. cruciata* induced on MS medium supplemented with 0.5 mg/l NAA and 2.0 mg/l BAP. (*J*) Plantlets of *G. pannonica* on MS medium. (*K*) Analysis on DNA content.

alive without callus formation. Figure 1 shows examples of the cytomorphological variation of callus types observed for all the *Gentiana* species investigated in this study. The six types of callus that could be distinguished in a PGR combination-dependent manner are the following:

1. Fine embryogenic callus: yellow in color with loose structure. The cells were round or slightly elongated and had divided both transversely and longitudinally (Fig. 1*A* and *A′*).
2. Nodule embryogenic callus: yellow colored, strongly hydrated, with loose nodular structure. The cells were round and contained numerous amyloplasts, dense cytoplasm, and centrally located nuclei (Fig. 1*B* and *B′*).
3. Filiform non-embryogenic callus: straw-yellow colored, hydrated, with rib- or thread-like structure. The cells were very elongated, divided only transversely, and contained a limited number of starch grain in plastids (Fig. 1*C* and *C′*).
4. White non-embryogenic callus: white colored, strongly adherent to leaf blade (Fig. 1*D* and *D′*).
5. Hydrated non-embryogenic callus: transparent or light yellow, highly hydrated. The cells were large, oval-shaped, and contained large vacuoles (Fig. 1*E* and *E′*).
6. Green non-embryogenic callus: green colored, compact structure. The cells contained numerous chloroplasts (Fig. 1*F* and *F′*).

G. kurroo, *G. cruciata*, *G. pannonica*, and *G. tibetica* produced five of the six callus types described above (excluding type 3), while *G. lutea* produced only hydrated non-embryogenic callus (type 5). *G. cruciata* formed not only the largest amount of callus but also callus tissue of the widest diversity. Filiform, non-embryonic callus was produced only from *G. cruciata* and *G. tibetica* leaf explants in the presence of Dic and 2,4-D. All species showed the highest intensity of callusing in the presence of Dic and produced the least amount of callus when the medium was supplemented with 2,4-D. The exception was *G. lutea*, which at a rate of about 16% created little lumps of light yellow callus (type 5) in the area of vascular bundle in the presence of NAA. Explants from these species on other media combinations failed to sustain callus growth and died.

Somatic embryos induction. A total of 189 different media combinations containing different types and levels of PGRs were tested for the ability to induce somatic embryos in five *Gentiana* species. Table 1 summarizes the PGR combinations capable of inducing somatic embryogenesis in each species. Somatic embryo formation was preceded by callus induction and was successfully induced from leaf explants of four out of the five *Gentiana* species investigated (Table 1). As for general callus production, *G. lutea* was the exception, and no somatic embryos were produced from this species under the culture conditions investigated.

In the presence of auxin and cytokinin, embryogenic centers emerged from the callus tissue (Fig. 2*A*). This process was observed after 5–6 wk of culture in all species. Somatic embryos were formed and developed through globular, heart (Fig. 2*B*), and cotyledonary (Fig. 2*C*) stages over an 8–10-d period and were generated only from the first and second types of callus described above (Fig. 1*A* and *B*). Depending on genotype and applied PGRs, several types of embryos were observed (Fig. 2*D–H*). Most possessed fused cotyledons, while some had hypocotyls and/or long rootlets. In *G. tibetica*, explants cultured on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l TDZ became highly vitrified (Fig. 2*F*).

NAA was more effective at inducing embryogenic tissues than 2,4-D or Dic, as four out of five species responded positively compared to only two or three in case of the latter auxin types (Table 1). This kind of morphogenic capacity occurred most intensively from explants of *G. kurroo* and occurred at all applied auxin and cytokinin

Table 2. Induction of somatic embryogenesis and number of embryos produced from leaf explants of 4 *Gentiana* species

	Species			
	G. kurroo	*G. cruciata*	*G. pannonica*	*G tibetica*
	1.0 mg/l NAA	1.0 mg/l NAA	0.5 mg/l NAA	2.0 mg/l NAA
	1.0 mg/l BAP	0.5 mg/l BAP	3.0 mg/l TDZ	3.0 mg/l TDZ
Frequency of SE %	45.9[a]	32.5[a]	9.9[a]	4.8[a]
No. of embryos per explant	14.2±8.3[b]	6.6±3.7[b]	15.7±3.2[b]	6.6±1.5[b]

Leaf explants were cultured on MS basal medium supplemented with the above growth regulators for 2 mo. in the dark at 22±1°C without subculture

SE Somatic embryogenesis

[a] Percentage of leaf explants producing embryogenic callus. Each value represents the mean result from 5 replicate Petri dishes.

[b] Mean ± SD number of somatic embryos per single responding explant obtained from 1 Petri dish.

combinations. Greatest average frequencies of embryogenic callus induction achieved in the present study were 54.7%, 32.5%, 13.2%, and 10.8% for *G. kurroo*, *G. cruciata*, *G. pannonica*, and *G. tibetica*, respectively, which occurred when leaf explants were cultured on 0.5 mg/l Dic + 1.0 mg/l Zeat, 1.0 mg/l NAA + 0.5 mg/l BAP, 2.0 mg/l NAA + 2.0 mg/l Kin, and 2.0 mg/l NAA + 0.25 mg/l CPPU, respectively. However, the best auxin/cytokinin level for this process was not always the best for production of the highest number of somatic embryos per explant (Table 2). Table 3 shows frequency of embryogenic callus induction for all combination of cytokinins with NAA. This process was strongly stimulated, especially when NAA was applied in combination with BAP or TDZ (Table 2). On the best media, the average number of somatic embryos from one explant ranged from 6.6 for *G. cruciata* and *G. tibetica* to about 15 for *G. pannonica* and *G. kurroo*. However, very high standard deviations were recorded in this response.

Root regeneration. Intensity of rhizogenesis from the explants depended on the species and the type of auxin supplied in the media. NAA stimulated the strong rooting response. In the case of *G. cruciata*, this auxin stimulated root induction in over 70% of the explants (Table 4). Rhizogenesis was not observed when explants were cultured on media containing Dic (Table 4).

Roots started to regenerate directly on the surface of leaf explants after 2–3 wk of culture (Fig. 3*A*) in *G. kurroo*, *G. cruciata*, and *G. tibetica* explants in the presence of NAA and Kin or Zeat. In all other cases, root induction was preceded by callusing (Fig. 3*B*), with subsequent rhizogenesis after 4–5 wk of culture. Roots were created from the fine embryogenic callus (type 1), nodule embryogenic callus (type 2), hydrated non-embryogenic callus (type 5), and green non-embryogenic callus (type 6). When embryogenic callus was produced, roots and somatic embryos were observed at the same time. Only explants of *G. lutea* were more rhizogenic than others in the presence of 2,4-D, especially in combination with TDZ (Table 4). Dependent on species and PGR combination, roots grew to various lengths in the range of 1.0 mm to more than 6.0 cm.

Induction of somatic embryogenesis from adventitious roots of primary explants. On the surface of adventitious roots, spontaneous somatic embryo regeneration was observed. This process was stimulated by the presence of various combinations of plant growth regulators and was greatest from tissues cultured on media containing either 2.0 mg/l NAA; 1.0 mg/l NAA + 0.25–0.5 mg/l Kin or 0.5–1.0 mg/l NAA + 0.5–1.0 mg/l BAP for *G. kurroo*, *G. pannonica*, and *G. cruciata*, respectively. Dedifferentiation of single cells of rhizodermis took place in the root elongation and

Table 3. Effect of NAA and cytokinin combinations on percentage somatic embryogenesis (%) for *Gentiana kurroo*, *G. cruciata*, *G. pannonica*, and *G. tibetica* leaf explants

Species	NAA (mg/l)	CYTOKININS (mg/l)																			
		Kin				Zeat				CPPU				BAP				TDZ			
		0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0	0.5	1.0	2.0	3.0	0.5	1.0	2.0	3.0
G. kurroo	0.5	1.3	0.0	2.7	2.5	1.5	9.2	10.0	6.4	0.0	0.0	0.0	0.0	11.2	13.6	9.2	2.0	17.1	34.0	22.9	20.0
	1.0	0.0	1.5	7.8	12.5	0.0	0.0	20.2	3.6	2.7	28.5	36.5	17.4	35.4	**45.9**	48.0	34.7	7.0	11.7	26.8	41.6
	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	1.9	2.2	36.7	14.7	0.0	14.0	7.6	0.0	1.5	12.3	19.4	0.0
G. cruciata	0.5	26.3	0.0	0.0	6.7	0.0	2.8	0.0	0.0	0.0	0.0	0.0	0.0	13.1	0.0	2.4	5.6	13.9	0.0	0.0	2.0
	1.0	9.1	2.6	0.0	0.0	5.4	2.1	2.0	1.9	0.0	0.0	0.0	0.0	**32.5**	12.8	0.0	0.0	3.6	0.0	0.0	3.0
	2.0	2.7	4.5	1.4	12.4	0.0	0.0	1.6	2.2	0.0	0.0	0.0	0.0	1.9	10.4	1.7	0.0	0.0	0.0	1.7	0.0
G. pannonica	0.5	1.8	0.0	7.8	0.0	5.0	1.0	0.0	1.3	1.0	3.4	1.9	0.0	0.0	1.1	0.7	2.2	1.3	4.0	4.5	**12.3**
	1.0	9.7	10.2	0.0	0.0	0.0	0.0	2.2	0.0	4.1	0.0	0.0	2.0	5.3	0.0	0.0	4.5	0.0	1.0	0.0	1.0
	2.0	8.8	0.0	11.6	13.2	5.4	2.0	1.0	0.0	0.0	0.0	0.0	0.0	10.8	7.0	11.5	2.0	0.0	0.0	0.0	0.0
G. tibetica	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.7	0.0	4.0	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.8
	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2	4.2	0.0	0.0	0.0	0.0	0.0	0.0	3.7	1.7
	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.8	3.2	5.9	9.6	0.0	0.0	1.7	6.1	0.0	2.8	5.9	**5.8**

Leaf explants were maintained on MS basal medium with the above combinations of growth regulators for 2 mo. in darkness at 22±1°C. Five Petri dishes were established for each auxin/cytokinin combination. Each result represents mean number of explants producing somatic embryos from 5 Petri dishes. Results were expressed as percentage value.
NAA 1-Naphthaleneacetic acid, *Zeat* zeatin, *Kin* 6-furfurylamonopurine, *BAP* 6-benzylaminopurine, *CPPU N*-(2-chloro-4-pyridyl)*N*′-phenylurea, *TDZ N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea
Bold numbers indicate the optimal value for somatic embryo production for each species

root hair zones (Fig. 3*C*), as well as in the area of the root cap (Fig. 3*D*), and led to formation of small cell clusters (Fig. 3*E*). Consecutive stages of the somatic embryo, the globular (Fig. 3*F*), heart (Fig. 3*G*), and cotyledonary stages were easily recognized (Fig. 3*H*). This morphogenic phenomenon is not included in the statistical assessment of somatic embryo production in leaf explant culture. However, frequency of this process in the presence of the aforementioned media compositions was regular and could become an important source of somatic embryos.

Maturation and conversion of somatic embryos. Somatic embryos maturated directly on the induction media. After 2 mo. of culture, somatic embryos were counted, and those at the cotyledonary stage were transferred into Petri dishes containing half strength MS medium (Fig. 2*I*) and then grown to reach plantlet stage on the same medium in jars (Fig. 2*J*). It took 2 wk for the embryos to develop to plantlets with two whorls of leaves. The conversion rate depended on induction media but was generally very high, reaching 100% in *G. kurroo*, *G. cruciata*, and *G. pannonica* (Table 5). Due to vitrification, only 42.8% of *G. tibetica* embryos passed conversion to produce plantlets.

DNA content. Multiplication of plants via *in vitro* cultures could be a very efficient method for plant production; however, somaclonal variation among the regenerants at the phenotypic, cytological, and molecular level could significantly reduce the value of such plants. Flow cytometry is a simple and efficient method for determination of nuclear DNA content and estimation of a plant's genome size. Despite the phenotypic appearance of some abnormalities among the somatic embryos, such as fused cotyledons, phenotypic variation was not noticed among the regenerated plantlets. The 155 plantlets obtained via somatic embryogenesis were examined by flow cytometry, and no changes of 2C value of nuclear DNA content in comparison to control plants was observed (Fig. 2*K*). These results indicate that application of auxin and cytokinin combinations, and the length of culture did not cause detectable phenotypic and cytological variation from regenerants in this study.

Discussion

Genotype is one of the most important factors influencing morphogenic response *in vitro*. Differences can be observed among species, cultivars, and individuals (Brown et al. 1995). Genotype specificity for embryogenic induction has been reported often in numerous species: *Crocus* sp. (Karamian 2004), *Zamia* sp. (Chavez et al. 1992), *Rosa* sp. (Kintzios et al. 1999), *Glycine max* (Bonacin et al. 2000), *Feijoa sellowiana* (Guerra et al. 2001), and *Carthamus tinctorius* (Mandal et al. 2001). In our work, among five investigated gentians, only *G. lutea* cultures did not undergo somatic embryogenesis. On the other hand, morphogenic capacity of *G. kurroo* was significant.

Genotype effect is also interactive with other culture factors such as PGR combinations. Among the three auxins tested, NAA was the best for induction of somatic embryogenesis, in a manner previously confirmed in *G. kurroo* (Sharma et al. 1993), *G. triflora*, and *G. triflora x scabra* (Hosokawa et al. 1996). Dicamba, in contradiction to NAA, stimulated only callusing. In *G. kurroo*, the callus was found to have a very high capacity for somatic embryogenesis. On the contrary, for *Eucalyptus globulus*, NAA stimulated only somatic embryogenesis, while Dic induced intensive rhizogenesis of leaf, cotyledon, hypocotyl, and stem explants (Pinto et al. 2002). For other gentians cultures, Dic has been successfully applied for maintenance

Table 4. Effect of growth regulators on percentage of rhizogenesis from leaf explants of 5 *Gentiana* species

Species	PGRs combinations used														
	2, 4 -D					NAA					DIC				
	Kin	Zeat	BAP	TDZ	CPPU	Kin	Zeat	BAP	TDZ	CPPU	Kin	Zeat	BAP	TDZ	CPPU
G. kurroo	0.5	0.4	0.0	0.0	0.0	33.8	22.2	26.9	40.6	36.2	0.0	0.0	0.0	0.0	0.0
G. cruciata	8.8	3.5	11.2	0.9	0.0	76.5	58.0	68.4	60.4	52.1	0.0	0.0	0.0	0.0	0.0
G. pannonica	1.3	0.5	0.7	0.3	0.7	59.0	55.1	53.7	32.6	52.1	0.0	0.0	0.0	0.0	0.0
G. tibetica	0.0	3.1	0.7	0.0	0.0	36.2	44.2	31.6	56.8	37.0	0.0	0.0	0.0	0.0	0.0
G. lutea	0.0	1.6	5.5	15.5	1.5	1.8	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Percentage of leaf explants producing roots for all combinations of auxin and cytokinin from five Petri dishes.
Leaf explants were cultured on MS basal medium supplemented with the above growth regulators for 2 mo. in the dark at 22±1°C without subculture.
The following concentrations of PGRs were used: 0.5, 1.0, and 2.0 mg/l of NAA (1-naphthaleneacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), and Dic (3,6-dichloro-o-anisic acid) in combination with 0.0, 0.25, 0.5, 1.0, 2.0 mg/l Kin (6-furfurylamonopurine), Zeat (zeatin), CPPU (*N*-(2-chloro-4-pyridyl)*N'*-phenylurea) or 0.0, 0.5, 1.0, 2.0, 3.0 mg/l BAP (6-benzylaminopurine) and TDZ (*N*-phenyl-N'-1,2,3-thiadiazol-5-ylurea).

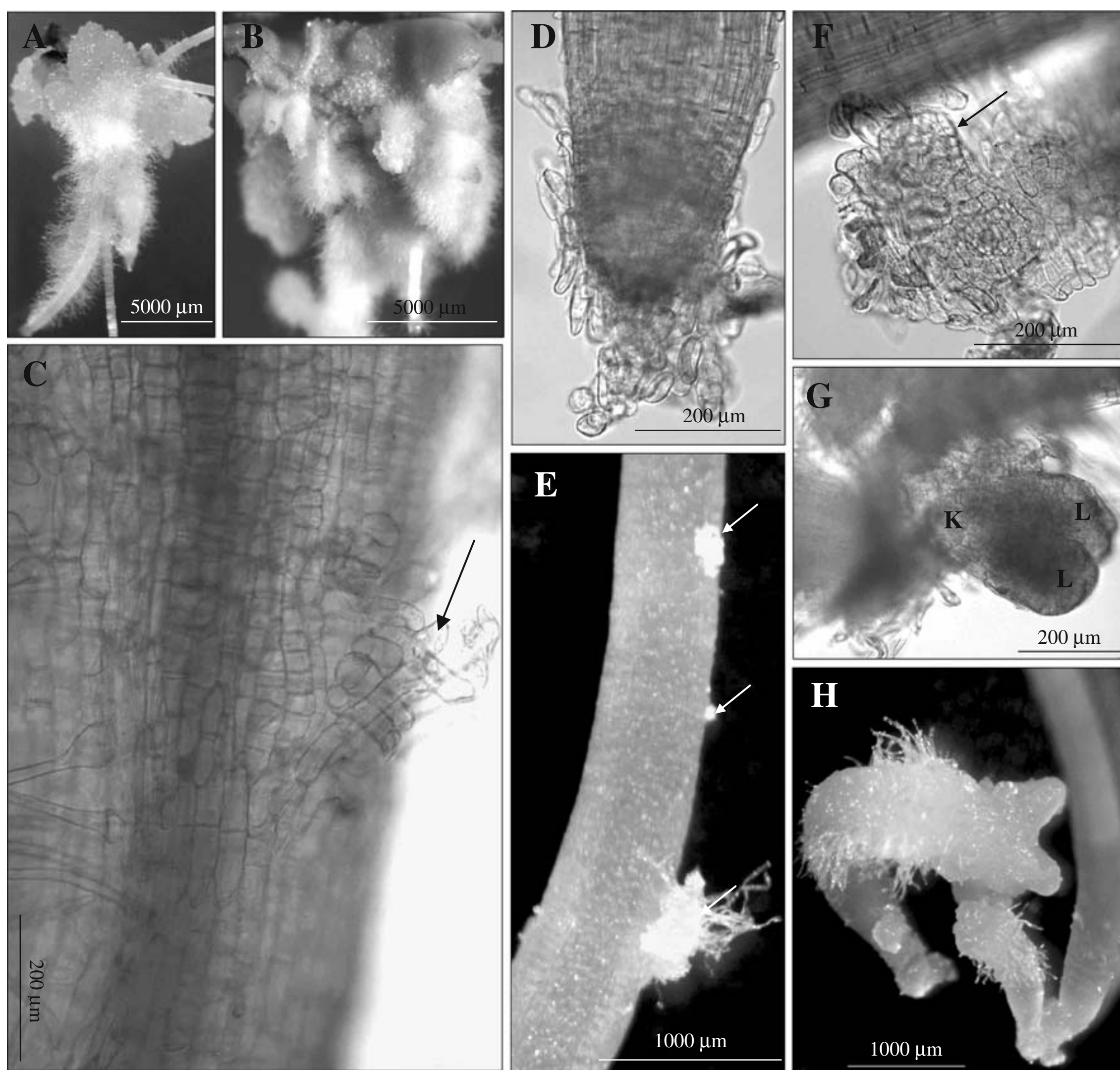

Figure 3. Somatic embryogenesis on adventitious roots regenerated from leaf cultures of *G. kurroo*. (*A*) Roots formed indirectly via callus. (*B*) Roots formed directly from abaxial side of leaf explant. (*C*) Differentiation of single cells of rhyzoderma in elongation zone (*arrow*). (*D*) Differentiation of single cells of rhyzoderma in root cap. (*E*) Formation of small amount of callus tissue (*arrows*). (*F*) Differentiation of globular embryos (*arrow*). (*G*) Somatic embryo in heart stage—visible root side (*K*) and cotyledons (*L*). (*H*) Somatic embryo at cotyledonary stage.

of cell suspension cultures with embryogenic capacities (Mikuła et al. 1996).

In the present study, a low level of morphogenic potential was stimulated by 2,4-D as few somatic embryos were observed in the presence of this auxin for *G. kurroo* and *G. cruciata* leaf explants. However, Bach and Pawłowska (2003) successfully used 2,4-D, as well as picloram for initiation of embryogenic callus on leaf explants of *Gentiana pneumonanthe*, but efficiency of somatic embryos was not reported.

On the basis of the performed experiments, we also could confirm that BAP and TDZ stimulated embryogenesis at high levels but did not always correlate with production of the largest amount of somatic embryos per explant. Variable numbers of somatic embryos produced per explant, as well as the presence of different developmental stages at the same time, indicate low synchrony of this process. Such asynchronous embryogenic induction is often observed and has been described for *Myrthus communis* (Canhoto et al. 1999), *Simmondsia chinensis* (Hamama et al. 2001), and *Epipremnum aureum* (Zhang et al. 2005).

Plants regenerated from somatic embryos produced from leaf explants of *Gentiana* were studied for cytological variations compared to the mother plant material. No such changes were detected at the cytological level despite some observed abnormalities in somatic embryo phenotype. High levels of vitrification in embryos of *G. tibetica* were observed which acted to reduce germination rates in this species (Table 5). For correct development of somatic

Table 5. Highest achieved conversion rates dependent on respective media for induction and maturation of somatic embryos in 4 *Gentiana* species

Species	Conversion rate (%)[a]	MS-based induction media supplemented with
G. kurroo	100.0	2.0 mg/l NAA + 2.0 mg/l TDZ
		2.0 mg/l NAA + 2.0 mg/l BAP
G. cruciata	100.0	1.0 mg/l NAA + 0.5 mg/l Kin
		0.5 mg/l NAA + 1.0 mg/l TDZ
G. pannonica	100.0	2.0 mg/l NAA + 0.5 g/l Zeat
		2.0 mg/l NAA + 1.0 mg/l Zeat
		2.0 mg/l NAA + 1.0 mg/l Kin
		2.0 mg/l NAA + 2.0 mg/l Kin
		2.0 mg/l NAA + 0.25 mg/l Kin
G tibetica	42.8	2.0 mg/l NAA + 3.0 mg/l BAP

[a] Leaf explants were cultured on half strength MS medium for 2 mo. at 22±1°C, 16-h light (100 μEm^{-2} s^{-1}), and 8-h dark without subculture. Percentage of conversion was counted for 100 somatic embryos; ten somatic embryos were transferred into each jar.

embryo regenerated from suspension cultures and seedling explants, GA_3 was required (Mikuła and Rybczyński 2001; Mikuła et al. 2005). In the present study, conversion of somatic embryos to plantlets was achieved efficiently (as high as 100%) and simply by subculture to media consisting of half strength MS medium devoid of PGRs. This confirms results of *G. pneumonanthe* and other species (Kurtén et al. 1990; Craig et al. 1997; Jain et al. 2002; Bach and Pawłowska 2003).

Production of somatic embryos was observed to occur from the surface of adventitious roots (Fig. 3*G*). This phenomenon deserves to be studied in more detail because of its potential application with *Agrobacterium*-based transformation technologies. To our knowledge, this is the first report concerning somatic embryogenesis from rhizodermal cells for Gentianaceae; however, the phenomenon was earlier described for *Cammelia japonica* (Vieitez et al. 1991).

In conclusion, genotypic and PGR combinations affected callus induction and somatic embryogenesis from leaf explants of five studied *Gentiana* species. Somatic embryogenesis was stimulated by NAA with BAP or TDZ. This system of plant regeneration via somatic embryogenesis has potential for micropropagation and for genetic transformation applications for these important species.

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