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Somatic embryogenesis of *Gentiana* genus I. The effect of the preculture treatment and primary explant origin on somatic embryogenesis of *Gentiana cruciata* (L.), *G. pannonica* (Scop.), and *G. tibetica* (King)

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Key words: Gentiana sp., preculture treatment, seedling explants, somatic embryogenesis, long term culture

Abstract

Experiments were carried out on three selected species of *Gentiana* genus: *Gentiana cruciata* (L.), *G. pannonica* (Scop.), and *G. tibetica* (King). Using MS medium supplemented with 2,4-D and kinetin a somatic embryogenesis system of plant regeneration was developed. Induction and intensity of somatic embryogenesis as the effect of integration of the following factors were studied, specifically: seedling pre-treatment (with and without GA_3 treatment), light condition (light versus the dark), and type of explant (root, cotyledon and hypocotyl). Numerous significant differences between studied factors were observed and statistically proved.

List of abbreviations: GA_3 – gibberellic acid, 2,4-D - dichlorophenoxyacetic acid, MS- Murashige and Skoog medium, Kin – kinetin, NAA – naphtaleneacetic acid, 6BA - 6-benzyladenine

Introduction

The family *Gentianaceae* includes 22 genus and little is known concerning tissue culture ability. *Gentiana*, comprising 361 species, is the largest genus in the family *Gentianaceae*, found mostly in the temperate and alpine regions of the word. The existing chromosome data show an extensive range of numerical variation: $2n = 12$ up to 96 (Yuan *et al.* 1996). Species of the genus have medicinal and horticultural value and are characterised by poor generative and vegetative multiplication *in vivo,* hence an effective system of *in vitro* regeneration is the most important prerequisite for plant genetic manipulation of the gentians. Only fourteen of 361 species of *Gentiana* genus have already been studied. For a majority of them clonal propagation based on stimulation of axillary bud development (Hosokawa *et al.* 1996, Lamproye *et al.* 1987, Momčilović *et al.* 1997) but only in case of *Gentiana scabra* did shoot regeneration occur in protoplast-derived callus cultures (Takahata and Jomori 1989).

Plant regeneration *via* somatic embryogenesis has certain advantages over organogenesis since embryos have both root and shoot meristems. Somatic embryogenesis is considered an efficient method for clonal propagation of genetically stable regenerants for many species but it is a laborious task.

There are only few papers concerning the process of somatic embryogenesis of *Gentianaceae* family and no one described direct embryogenesis. In the majority of studies embryogenic suspension was initiated by callus tissue cultures derived from seedling explants: hypocotyl, cotyledon, root (Mikuła *et al.* 1996), leaf blade and apical meristem of *in vitro* axenic plantlet or flower bud (Ørnstrup *et al.* 1993). Cytomorphological studies of somatic embryogenesis of *G. tibetica* and *G. cruciata* revealed numerous morphological disturbances which decreased the yield of regenerants (Mikuła *et al.* 1996).

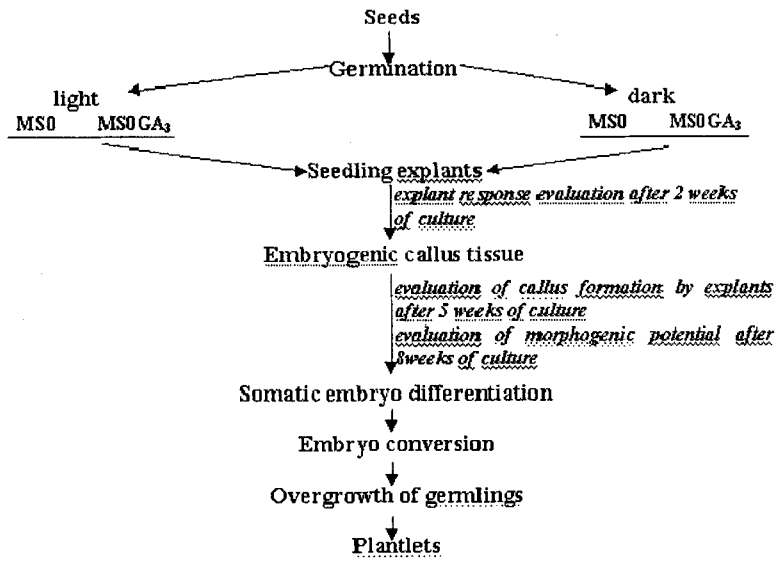

Fig. 1. Schema of the experiment design

Plants of *G. grassicaulis* were regenerated by embryogenic callus derived from protoplast cultures *via* somatic embrygenesis on hormone-free MS medium (Meng *et al.* 1996).

Due to the lack of analysis of somatic embryogenesis of *Gentiana* we would like to present a series of papers describing our experiments which were initiated in 1995. Somatic embryogenesis is a complicated system of plant regeneration which can be controlled by numerous factors: the genetic background of plant material, the manipulation of primary explant initiating culture and culture conditions.

The aim of the presented paper is to show the induction and intensity of somatic embryogenesis for three species (*G. cruciata, G. pannonica, G. tibetica*). The effect of seedling pre-treatment by GA_3, its culture in light and in dark, and type of explant (root, cotyledon, hypocotyl) on this morphogenic event are studied and discussed.

Material and Methods

Plant material

Experiments were carried out on three selected species *Gentiana cruciata* (L.), *G. pannonica* (Scop.), and *G. tibetica* (King) and Fig. 1 explains the design of experiments. Seeds were collected from field grown plants in the medicinal and aromatic plant collection of Botanical Garden – Center for Biological Diversity Conservation, Polish Academy of Sciences in Warsaw. Non-harmed, fully developed mature seeds were sorted out using OLYMPUS dissecting microscope. Seeds were surface sterilised by immersing in 70 % (vol/vol) ethanol for 30 s followed by washing with 0.1 % $HgCl_2$ supplemented with two drops of Tween 80 for 15 min. and finally rinsed five times with sterile bi-distelled water. Respectively seeds were stratificated on wet white paper at temperature 3 - 5 °C for 14 days. Seeds were germinated in light and in the dark in the presence of two media: 1) MS0 medium - Murashige and Skoog (Murashige and Skoog 1962) hormone free medium contained only half concentration of macro- and microelements and full strain of vitamins, 2) $MS0GA_3$ medium - MS0 medium supplemented with 1.0 $mg \cdot l^{-1}$ GA_3. Each treatment contained a total of 200 seeds.

All seedling cultures were maintained in the culture room at temperature 21±1 °C and with a light intensity of 25 $\mu E^{-2} \cdot s^{-1}$ with photoperiod 16/8 hrs day/night.

Culture initiation and maintaining

Explants *i.e.* cotyledons, hypocotyls and roots originated from 21 day-old seedlings of three studied species. Seedlings, originated from seeds germinated in the dark five days before explant isolation were transferred to the light conditions (see above). Each of seedling used as explant sources was divided with the following pattern: cotyledons, hypocotyl (split into two parts: under cotyledonary and over root-shoot junction with the size not longer than 3 to 6 mm) and whole root.

Cultures were maintained in 9 cm wide plastic petri plate with 25.0 ml of induction medium (IM) and sealed with parafilm. IM - medium was composed by MS (Murashige and Skoog 1962) medium supplemented with 0.5 $mg \cdot l^{-1}$ 2,4-D + 1.0 $mg \cdot l^{-1}$ Kin + 30.0 $mg \cdot l^{-1}$ sucrose and solidified with 0.8 % Difco-Bactoagar. Before autoclaving medium was adjusted to pH 5.8. Fifteen explants originated from three seedlings were plated on the medium in one petri plate. One petri plate was considered as one replication. For each studied species experiments had 44 replications for both light and dark culture of seedling.

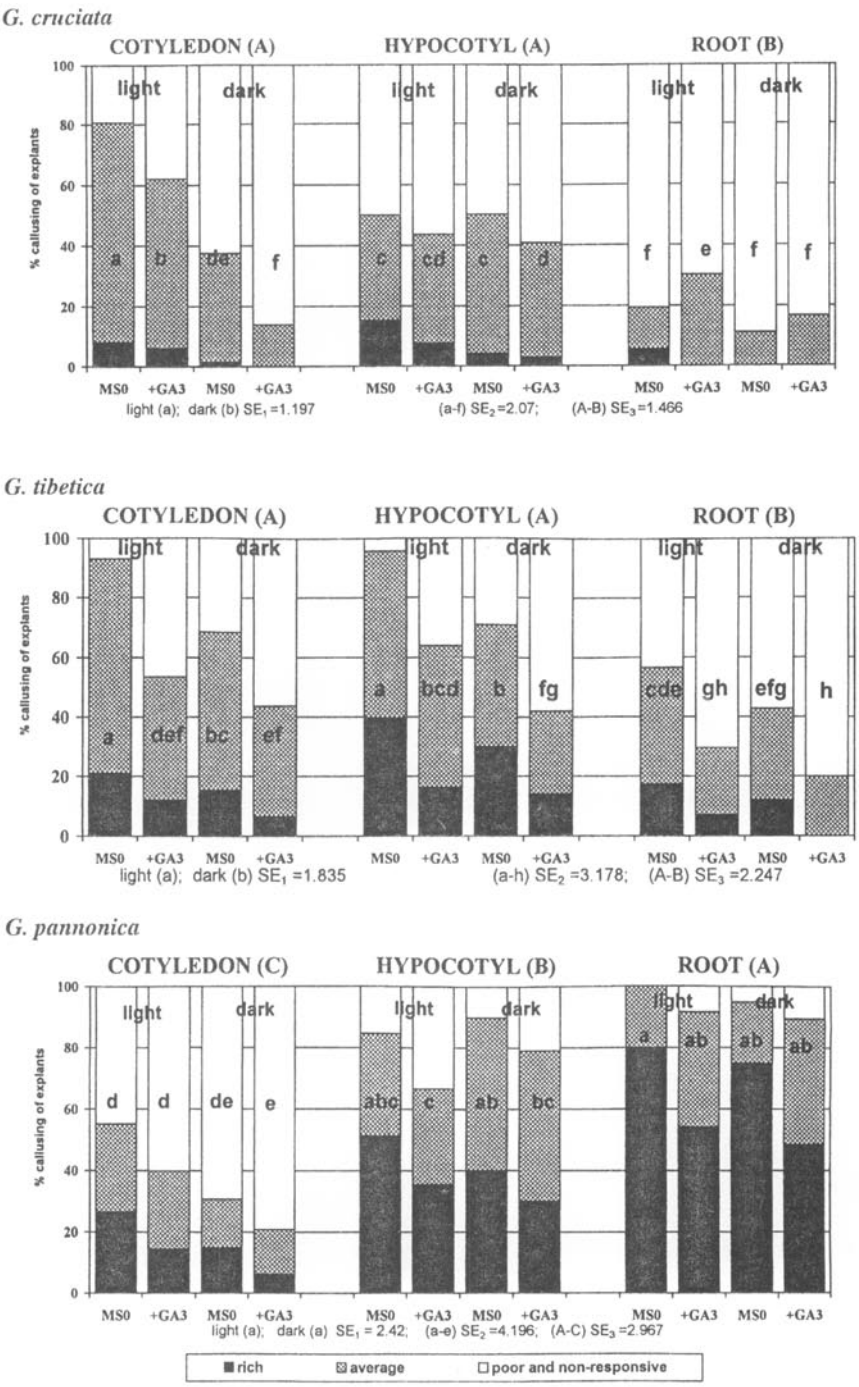

Fig. 2. The effect of seed germination conditions and seedlings growth on callus production intensity (after 2 weeks)*
cotyledon/hypocotyl/root - seedling explants
light or dark - germination of seeds and growth of seedlings in light or in darkness
MSO - germination of seeds and growth of seedlings on the medium 0.5 MSO
+GA_3 - germination of seeds and growth of seedlings on the medium 0.5 MSO + 1.0 $mg \cdot l^{-1}$ GA_3
* analysis of three-factor ANOVA of variance. Significant at the 5 % level. SE_1 - standard error for conditions of lighting; SE_2 - hormones; SE_3 - origin of explants

Callus tissue induction and somatic embryogenesis evaluation

Due to the slow callus development, the description of morphogenic capability of particular explant was evaluated during 10 week-long culture. The primary assessment was the initial explant response after two weeks of culture due to condition of seedling germination. The intensity of callus formation and proliferation was visually evaluated as follows: rich, average, poor and non-responsive (Fig. 2).

After 5 weeks of culture in light or in the dark, the second evaluation of explants for the capability to form embryogenic callus was done The same scale of evaluation was assumed (Fig. 3). Morphogenic capacity of the particular explants was determined by the number of somatic embryo formed after 8 weeks of culture. Results present an average of 30 explants.

Somatic embryo conversion and plantlet regeneration

Single or clusters of somatic embryos with small amount of embryogenic callus were transferred to conversion medium. To assess the optimal conversion medium following concentrations of plant growth hormones were tested: 0.1 and 0.5 $mg \cdot l^{-1}$ GA_3 and, 0.1, 0.5 and 1.0 $mg \cdot l^{-1}$ both Kin and NAA supplemented with 40.0 $g \cdot l^{-1}$ sucrose. The optimal medium was selected on the base of leaves and root

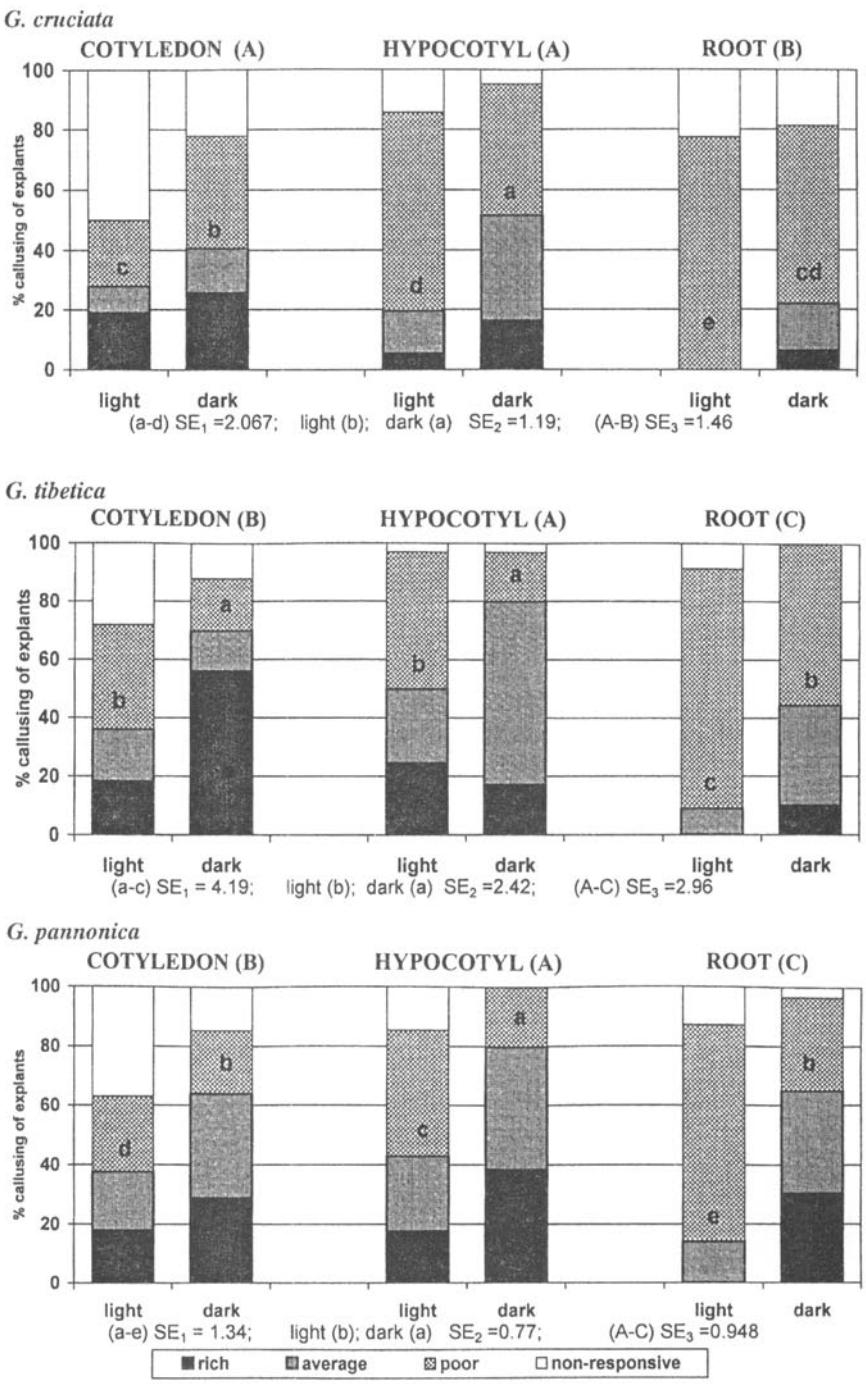

Fig. 3. The effect of culture conditions and type of explant on callus production intensity (after 5 weeks)*
light or dark - cultures carried in light or in darkness
* analysis of one-way ANOVA of variance for comparison middles of callusing explants (SE_1) and the analysis of two-factor of variance for correlation between comparison of lighting(SE_2) and origin explants (SE_3). SE - standard error. Significant at the 5 % level

primordia development of somatic embryo. The conversion abilities were expressed on the percentage of embryos capable to form germlings. Usually 400 embryos being in cotyledonary stage were

plated. Forty embryos plated in one petri plate were considered as one replication.

Small germlings with two leaflets and root were transferred from conversion to germling maintaining 05MS (half concentration of minerals of MS medium) medium supplemented with 10.0 $g \cdot l^{-1}$ sucrose to stimulate their growth. Plant hardening occurred in jars fill with perlite soaked with 05MS medium free of sucrose and plant growth hormones. After one month of growth, plants that were 4 cm long with good root system were transferred to the pot containing mixture of peat and sand.

Statistical analysis

All data scored were evaluated by analysis of variance using Type III Sums of Squares (STATGRAPHICS Plus 2.1 for Windows). The least significant differences (LSD) 5 % probability between the means were evaluated by Fisher's test.

Results

GA_3 and light effect on seed germination and growth of seedling

In control cultures on MS0 medium seeds of studied species germinated very slowly and irregularly. The average of germination after 21 days of culture was of 89 % and only 66.7 % of seedlings were well developed. GA_3 decreased the number of germinating seeds (Table 1) but increased the seedling uniformity which facilitated next steps of the experiment. This result was observed for both light and the dark cultures. Seedlings cultured in the presence of light developed cotyledons, shortened hypocotyl (not longer than 6 mm) and root. Those coming from the dark had weaker cotyledons, and root and hypocotyl more elongated. GA_3 additionally stimulated elongation of hypocotyl, almost twice the length of the control, even when cotyledons were still poorly developed. Table 2 shows GA_3 and light effect on uniform growth of seedlings.

Intensity of callus tissue proliferation

During first few days of culture, all explants swelled due to intensive cell proliferation. Callus originated from cultures in dark had a yellowish appearance and loose structure. In some cases, callus proliferation was accompanied by the transparent and watery matrix with numerous single small cells. In cultures maintained in light explants proliferated callus that was compact and initially a yellowish one, but which turned green after 4 - 5 weeks of culture although never expressed embryogenic competence.

Explants originated from morphologically not uniform seedlings implanted on callus induction medium responded differently in initiation and formation of calli. For three studied species the cotyledon explants formed callus on surface of cutting and in areas of damaged epidermis. Non-responding parts of the explant turned brown and finally died (Plate 1a). The hypocotyl explant responded similarly, but callus proliferation occurred on both ends of the explant. Experiment employed two explants of each hypocotyl and the one that located closer to cotyledonary node expressed higher callus production competence than the one nearest root – shoot junction (Plate 1b). Strong species specific response of

Table 1. Effect of GA_3 and light conditions on seed germination on agar medium after two weeks of culture

Species	Seed germination *(%)			
	light		darkness	
	MS0	+GA_3	MS0	+GA_3
G. cruciata	94.0 ± 3.29	78.0 ± 5.74	90.0 ± 4.16	88.0 ± 4.50
G. tibetica	90.0 ± 4.16	65.0 ± 6.61	90.0 ± 4.16	75.0 ± 6.00
G. pannonica	90.0 ± 4.16	76.0 ± 5.92	81.0 ± 5.44	82.0 ± 5.32
Average	91.3	73	87	81.7

± SD - standard deviation

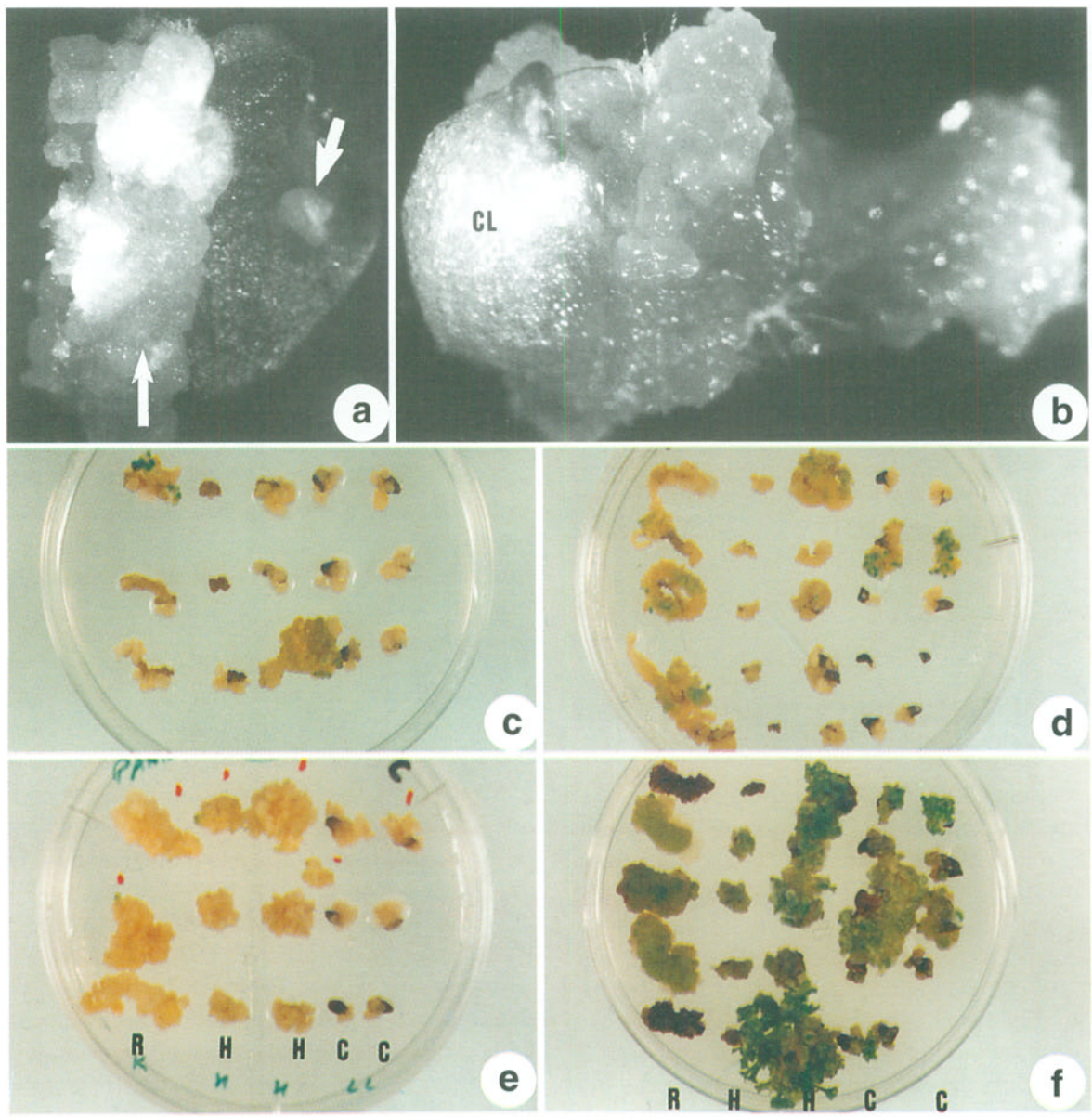

Plate 1. Primary response of *Gentiana* sp. seedling explants on induction agar medium (MS medium supplemented with 0.5 $mg \cdot l^{-1}$ 2,4-D + 1.0 $mg \cdot l^{-1}$ Kin)
a) callus proliferation by cotyledon and
b) by hypocotyl explant after two weeks of culture
c) and d) response of particular explants of *Gentiana cruciata* after 5 weeks of culture (c. - in light, d. - in the dark)
e) and f) response of *G. pannonica* explants after 5 weeks of culture (e.- in light, f. - in the dark)
R - root; C - cotyledon; H - hypocotyl , Cl – cotyledonary node

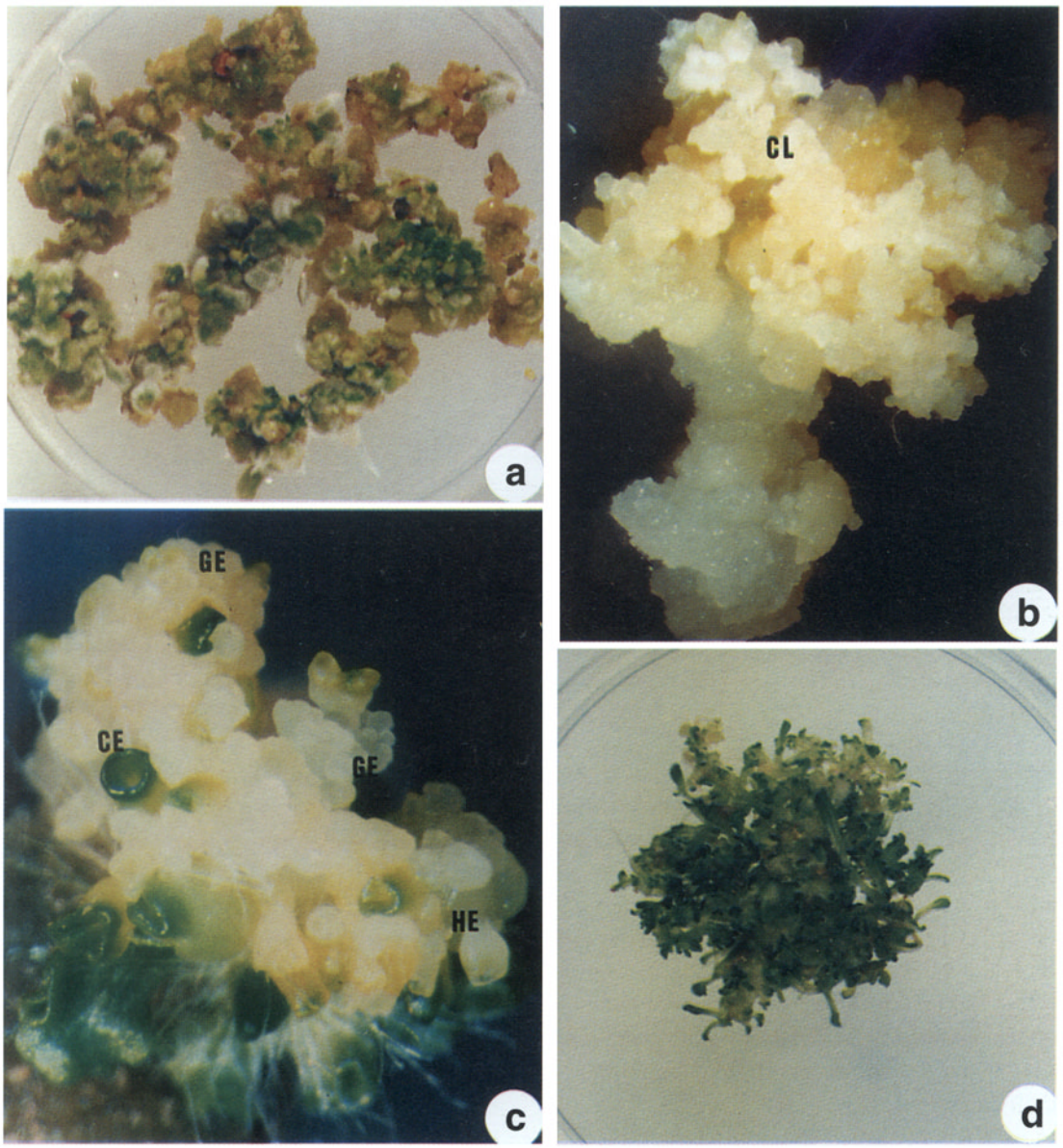

Plate 2. Non- and embryogenic cultures of *Gentiana* sp. on induction agar medium (MS medium supplemented with 0.5 $mg \cdot l^{-1}$ 2,4-D + 1.0 $mg \cdot l^{-1}$ Kin)
a) compact greenish and non-embryogenic callus tissue with numerous short roots after 10 weeks of culture in the light
b) intensive embryogenic tissue regeneration connected with cotyledonary part of hypocotyl explant in the dark
c) somatic embryos in various stage of development after 6 weeks of culture on initial medium
d) numerous small germlings as the result of rich somatic embryo conversion on MS medium supplemented with 0.5 $mg \cdot l^{-1}$ GA_3 + 0.5 $mg \cdot l^{-1}$ NAA+ 0.5 or 1.0 $mg \cdot l^{-1}$ Kin.
GE - globular embryo, HE - heart embryo, CE - cotyledonary stage of embryo, CL - cotyledonary part of hypocotyl

Table 2. Effect of GA_3 and light on uniform growth of seedling on agar medium after 5 weeks of culture

Species	Developed seedlings (%) *			
	light		darkness	
	MS0	+GA_3	MS0	+GA_3
G. cruciata	71.80 ± 6.05	92.30 ± 4.18	64.44 ± 6.99	87.50 ± 4.89
G. tibetica	67.78 ± 6.28	90.80 ± 4.98	65.56 ± 6.94	86.67 ± 5.44
G. pannonica	64.40 ± 6.58	88.16 ± 5.14	66.05 ± 7.29	87.80 ± 5.01
Average	68	90.4	65.5	87.3

*after 21days of culture, ± SD - standard deviation

root explants was observed. *G. cruciata* roots formed callus on both ends of explant: on apical meristem and opposite the cut surface. During the culture, callus was formed along the explant from the ends. In culture of *G. pannonica* and *G. tibetica* root explant responded more intensively and callus proliferation regularly covered the whole explant, especially intensively in the case of cultures maintained in the dark.

After two weeks of culture (Fig. 1), the primary response of the explant was evaluated with regard of GA_3, light and the dark treatments during seed germination, and seedling growth. The most intensive callus proliferation occurred on explants originated from seedlings growing in the light. A significantly higher effect of light compared to the dark treatment on callus proliferation by explants of *G. cruciata* and *G. tibetica* was ascertained. The treatment of seeds by GA_3 affected on callus formation. An especially negative effect of GA_3 treatment was observed in case of callus formation from cotyledons originated from seedlings growing in the dark. The cotyledon explants of *G. cruciata* and *G. tibetica* with *G. pannonica* responded almost 6 and two times less than explants originated from seedlings cultured on light without GA_3 treatment, respectively. All explants of *G. tibetica* isolated from seedling growing in the dark with the GA_3 treatment were able to form 50 % less callus comparing to those growing in light but not treated with GA_3 (Plate 1b). Only in root explant cultures of *G. cruciata* in both light and the dark a stimulatory effect of GA_3 on callus proliferation was observed. Using three factorial analysis of variance we did not find differences for callus formation by cotyledon and hypocotyl explants of *G. cruciata* and *G. tibetica*. Significant differences among explants of *G.pannonica* were proved and the best callus proliferation follows: root, hypocotyl and cotyledon explants.

After 5 weeks of culture (Fig. 2), the next assessment of callus proliferation competence revealed a significant negative influence of light for all explants and species, however callus formation intensity was correlated with the type of explants and with studied species. *G. tibetica* and *G. pannonica* explants proliferated more intensively and callus growth was higher than in the case of *G. cruciata* explants (Plate 1c, d). Cotyledons of *G. tibetica* and *G. pannonica* in the dark treatment proliferated intensively with average about 60 - 70 % when in light only 40 %, but *G. cruciata* explants responded 40 % and 30 %, respectively. In case of hypocotyl explants the following response was observed: for *G. tibetica* and *G. pannonica* in the dark 80 %, in light 40-50 % (Plate 1e, f). For *G. cruciata* 50 % (in the dark) and 20 % (in light) callus proliferation was recognised (Plate 2a, b). For root explants the best response was presented by *G. pannonica* and about 65 % of explants produced callus intensively in the dark and only 20 % in light. In the same conditions *G. tibetica* responded only in 40 % and 10 %, respectively. Only 20 % of root explants of *G. cruciata* formed callus in the dark, while in light 80 % of explants proliferated callus very poorly. Two factorial analysis of variance revealed that both type of explants and light conditions had significant effects on callus tissue proliferation. We did not find a significant interaction of both studied factors. The most intensive callus proliferation was pre-

Table 3. Number of somatic embryos originated from single explants after 8 weeks of light culture

Species	Type of explant		
	Cotyledon	Hypocotyl	Root
G. cruciata	39 ± 6.72	46 ± 6.77	0
G. tibetica	98 ± 14.06	111 ± 16.09	0
G. pannonica	59 ± 10.15	71 ± 9.34	17 ± 4.43

± SD - standard deviation, data present average of 30 explants

sented by hypocotyl and cotyledon explants of *G. tibetica*.

Somatic embryogenesis and plant regeneration

The callus induction medium composed of 0.5 $mg \cdot l^{-1}$ 2,4-D and 1.0 $mg \cdot l^{-1}$ Kin stimulated somatic embryo formation. In both light and dark cultures the process of somatic embryogenesis was observed. In light cultures the first embryogenic centres were found in the fifth week of culture. One week later dissecting microscope analysis gave evidences of the progress of somatic embryogenesis. In this time somatic embryos reached globular and more progressed stages up to early cotyledonary stage. (Plate 2 b, c). In cultures maintained in the dark embryogenesiss was about 2 weeks delayed. The extension of the culture led to the loss of embryogenic callus tissue because of very intensive embryo production. Earlier transferring some pieces of this callus tissue to liquid medium allowed us to develop cell suspension culture and keep its embryogenic competence for at least four years.

For all studied species, hypocotyl explants demonstrated the highest embryogenic competence. Statistical analysis did not bring significant differences for somatic embryos production for cotyledon and root derived explants within each species. However, significant differences for somatic embryogenesis among studied genotypes were found. In cultures of *G. tibetica* and *G. cruciata* the highest and the lowest number of somatic embryos was achieved, respectively. Among root-derived cultures only *G. pannonica* culture produced somatic embryos and it had the lowest average per explant (Table 3).

Table. 4. Effectivity of somatic embryo conversion on medium MS + 0.5$mg \cdot l^{-1}$ GA_3 + 1.0 $mg \cdot l^{-1}$ Kin + 0.5 $mg \cdot l^{-1}$ NAA + 40.0 $g \cdot l^{-1}$ sucrose

Species	No. of implanted embryos	No. of converted embryos	Convertion (%)
G. cruciata	400	216	54 ± 4.88
G. pannonica	400	252	63 ± 4.73
G. tibetica	400	268	67 ± 4.61

± SD - standard deviation

Somatic embryogenesis led not only to fully formed embryos, but also to some morphological disturbances. Table 4 shows the percentage of selected, morphologically non-disturbed embryos which converted (Plate 2d) on medium supplemented by GA_3, Kin, NAA and elevated concentration of sucrose. Germlings required two steps of the hardening as follows: first - transferring to 05MS agar medium and second - culturing in perlite supplemented with liquid 05MS salt medium. From one gram of tissue about 300 plantlets were successfully transferred from *in vitro* to greenhouse conditions.

Discussion

The limited data on somatic embryogenesis of gentians indicated the necessity to initiate intensive studies of this phenomenon for selected species of this genus. In this paper, the various conditions which affected the initiation of somatic embryogenesis were described.

Under selected conditions, species specific responses of studied gentians were proved. Among three species *G. tibetica* appeared to be the most responsive genotype for cotyledon and hypocotyl explants. Poor response of all explants in both light and the dark culture conditions characterised *G. cruciata*. On the contrary *Gentiana pannonica* expressed its high morphogenic competence for three

studied explants, from which long term embryogenic culture were developed (Mikuła *et al.* in press).

Cultures were initiated by the seed sowing on solidified medium supplemented with GA_3. Significant differences in seed germination *in vitro* of the three studied species were observed. Cultures in the dark and in the presence of GA_3 show more significant differences among species than in culture condition combining light and GA_3. It is known that seed germination of many *Gentiana* species require the chemical or physical treatment to break dormancy. It was already shown that the seed dormancy breaking of *G. scabra* (Bicknell 1984), *G. cerina* and *G. corymbifera* (Morgan *et al.* 1997) required GA_3 with maximum concentration 100 $mg \cdot l^{-1}$. However, higher GA_3 concentration can suppress seed germination. Because of the lasting effect of GA_3, this study describes its effect on the morphogenic abilities of *Gentiana* cultures. In this paper GA_3 concentration used affected seed germination, growth of seedlings and indirectly explant callus proliferation. The presence of GA_3 caused the seeds of all species to germinate less than the untreated control in both dark and light, which was contrary to the previously described results with *G. cerina* and *G. corymbifera* (Morgan *et al.* 1997). In our experiments GA_3 affected seedling uniformity, which helped to design and conduct experiments. Both, elongation of hypocotyl (Grubisic *et al.* 1995) and weaker development of cotyledons were also observed. Although hypocotyl tissue was affected by GA_3, it did not affect the quantity of derived callus from the explant of the studied organs. A negative correlation between the stimulatory effect of GA_3 on the growth of explants and callus proliferation was found. Conditions of germination such as: darkness and GA_3 treatment significantly inhibited development of cotyledons, which affected callus proliferation response. It is known that GA_3 plays a very specific role in the induction and maintenance of *in vitro* cultures. In carrot suspension cultures GA_3 appeared to be the inhibitor of somatic embryogenesis (Fujimura and Komamine 1975), however, for tobacco cultures it stimulated callus proliferation (Starling *et al.* 1986) and inhibited meristematic layer formation (Murashige 1964). In cultures of gentians and related species, GA_3 affects the shoots elongation and their multiplication (Morgan *et al.* 1997) but it is not clear why shoot explants of *G. cerina* produced more roots than control when medium contained 1.0 $mg \cdot l^{-1}$ GA_3 (Morgan *et al.* 1997).

The intensity of embryogenic callus proliferation was affected by the type of explant. The most responsive explant was hypocotyl and then the cotyledon. Root cultures of *G. cruciata* did not respond well. Here greater attention was given to embryogenic cultures developed from root of *G. pannonica*. The intensity of *G. pannonica* root-derived culture proliferation developed in the dark was comparable to effectiveness of callus formation of cotyledon (in the dark) of *G. pannonica*, but hypocotyl and cotyledon (both in the dark) of *G.tibetica*. In all already published papers on gentiana somatic embryogenesis, medium manipulation consisted of the explant tissue treatment by auxin (2,4-D) to induce cell division, followed by the transfer of proliferating tissue to hormone free medium to develop embryo (Ørnstrup *et al.* 1993, Tariba 1994). Contrary to these results our cultures regenerated embryogenic callus on the same medium (2,4-D and Kin) that formed embryos. The data suggested that 2,4-D level used was sufficient to initiate cell division and embryo development up to cotyledonary stage. We should stress that embryogenic tissue completely decayed on initiation medium (because of intensive embryo production) and only by its transferring to liquid medium could this loss in activity be prevented. Involvement of more plant growth hormones in somatic embryogenesis system was described for *Gentiana crassicaulis*. Initially protocolonies were maintained on medium supplemented with 3.0 $mg \cdot l^{-1}$ zeatin, 2.0 $mg \cdot l^{-1}$ 6BA, 1.0 $mg \cdot l^{-1}$ GA_3 and 1.0 $mg \cdot l^{-1}$ NAA. Later protocolonies were transferred to media lacking plant growth regulators which stimulated the protoplast-derived callus to become embryogenic. Finally, after additional selection of cultured tissue, somatic embryos were produced (Meng *et al.* 1996).

Plant regeneration *via* somatic embryogenesis follows specific developmental and morphological steps which result in well formed germlings. For three studied species, to develop proper conditions for conversion of the embryo to germlnig a medium supple-

mented with GA_3 + Kin + NAA was used. The best medium for embryo conversion contained 0.5 mg l^{-1} GA_3 + 0.5 mg l^{-1} NAA+ 0.5 or 1.0 mg l^{-1} Kin. The medium stimulated the correct development of young leaves and roots of germling. Statistical analysis revealed differences among studied species and *G. cruciata* produced significantly less germlings than the other studied species.

In conclusion we have described a system for initiating cultures which were competent to regenerate plants *via* somatic embryo formation and to develop long-term embryogenic suspension cultures. Suspensions will be used for studies of various aspects of somatic embryogenesis in liquid medium.

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