Chapter 26

Somatic embryogenesis of *Gentiana* genus IV.: Characterisation of *Gentiana cruciata* and *Gentiana tibetica* embryogenic cell suspensions

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Abstract: Experiments to characterise long-term embryogenic suspension cultures of *Gentiana cruciata* (L.) and *G. tibetica* (King) are reported. Cell suspensions of both species differed in the percentage of five selected fractions of cell aggregates, as well as in fresh and dry mass during three years of culture. In *G. tibetica* the ratio of cells in phase G_2 to G_1 was higher than in *G. cruciata*. The response of suspension cultures to GA_3 (at 0, 1.49 or 2.89 µmol), kinetin (at 0, 2.32, 4.64 or 9.28 µmol) and adenine sulphate (at 0 or 434 µmol) was studied. The increase of kinetin concentration stimulated embryo production in suspensions of *G. tibetica*. Somatic embryo production in *G. tibetica* was significantly higher than in *G. cruciata*. In *G. tibetica*, the aggregate fraction >450 µm was at least four times more productive than the same fraction in *G. cruciata* suspensions.

Key words: cell aggregate, flow cytometry, gentian, long-term suspension culture, plant growth regulators, somatic embryo

Abbreviations: AS - adenine sulphate; BAP – benzylaminopurine; Dic – dicamba; DW – dry weight; ECM - embryo conversion medium; FW – fresh weight; GA_3 - gibberellic acid; G_1 and G_2 phases of cell cycle; IM – initiation medium; KIN - kinetin; MM – maintaining medium MS – Murashige and Skoog medium; NAA – naphthaleneacetic acid; PEM – proembryogenic mass; PGR - plant growth regulator; TDZ - thidiazuron

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1. Introduction

Most gentians originate from alpine climatic zones: about 190 species were discovered in the Himalayas and Alps, and 27 in the Pyrenees. More than 100 species are protected by law in their native habitats. Many gentians are used in gardens as ornamental plants, some species have also pharmaceutical value. Because of the various uses of *Gentiana* species, highly efficient methods for their propagation are required.

Micropropagation of gentians is usually *via* organogenesis (Mikuła and Rybczyński, 1999): shoot or axillary meristem cultures are initiated from *in vivo* or *in vitro* cultured plants. Somatic embryogenesis appears to be an effective system of plant propagation in tissue cultures, already described in three *Gentiana* species: *G. cruciata*, *G. pannonica* and *G. tibetica* (Mikuła and Rybczyński, 2001). The initiation (Mikuła and Rybczyński, 2001;Mikuła et al., 2002b; Mikuła et al., 2003), proliferation and maintenance of embryogenic cell suspension cultures of gentians (Mikuła et al., 2001; 2002a) have been described in our previous papers. Embryogenic cell suspensions appear to be more productive than callus cultures of gentians grown on gelled medium, and provide a long-term source of somatic embryos for plant regeneration (Mikuła et al., 2002).

Many trials have been undertaken to obtain genetically modified gentians – new ornamental varieties and plant material for secondary metabolite production (Momčilović et al., 1997). Long-term embryogenic suspension cultures can provide protoplasts for somatic hybridisation and transformation. The long-term preservation of suspension cultures in liquid nitrogen (-196°C) creates opportunities for experiments to be carried out on the same plant material during several years.

The aim of this experiment was to characterise the well-established highly embryogenic cell suspensions of *Gentiana cruciata* and *G. tibetica*. Selected growth parameters of the suspension cultures were evaluated. Additionally, the influence of different PGRs applied in the gelled medium on PEM development and embryo production was studied.

2. Material and methods

Callus cultures of *G. cruciata* (L) and *G. tibetica* (King) were induced on agar-gelled MS (Murashige and Skoog, 1962) supplemented with 4.52 µmol 2,4-D and 0.53 µmol KIN (IM) (Mikuła et al., 1996b; Mikuła and Rybczyński, 2001). After six months, callus pieces were transferred to liquid MM composed of MS mineral salts and supplemented with 4.52 µmol Dic, 0.45 µmol NAA, 5.77 µmol BAP and 434 µmol AS. Cultures were

maintained under continuous diffused light (3.5 μE m^{-2} s^{-1}) at 23°C on an INFORS gyratory shaker at 130 rpm. Each 250 ml Erlenmeyer flask contained 80 ml of the cell suspension.

Studies on the cell cycle, the percentage of cell aggregate fractions, the fresh and dry mass of cultures as well as flow cytometric analysis and light and scanning electron microscopic examinations, were carried out to characterise the established cell suspensions. The methods described in the previous paper were used (Mikuła et al., 2002).

To characterise the cell suspensions, their morphogenic potential in different culture conditions was additionally taken into consideration. To study the effect of selected PGRs on gentian somatic embryogenesis, three-year-old suspensions were implanted onto the agar-gelled MS supplemented with GA_3 (at 0, 1.49 or 2.89μmol), KIN (at 0, 2.32, 4.64 or 9.28 μmol) and AS (at 0 or 434 μmol). Before the implantation, cell aggregates were divided into two fractions depending on their size (240-450 μm and >450 μm).

The effect of cell aggregate fraction on somatic embryo production was analysed by means of 1-factor variance analysis, whereas the effect of GA_3 and KIN - by 2-factor analysis (ANOVA).

3. Results

In the MM supplemented with Dic, NAA, BAP, and AS, proembryogenic masses of *G. cruciata* underwent cyclic morphogenic changes. Single embryogenic cells and small cell aggregates (phase I) (Figure 1a) formed larger aggregates with proembryos and somatic embryos at the globular stage (phase II) (Figure 1b, c). Under the same culture conditions the embryos developed to phase III, initiated by the degradation of the proepidermal cells (Figure 1d). Finally the structure disintegrated into aggregates and single cells. The particular phases followed each other during the two-week subculture period. The phenomenon described above has not been observed in *G. tibetica* cell suspensions. Further somatic embryo development required implantation of the cultures onto the agar-gelled medium with the same PGRs.

Growth phase strongly affected the regeneration competence of PEM in *G. cruciata*. Cell aggregates of 240-450 μm (phase I and II) produced *c.*200 somatic embryos from 100 mg FW of tissue when only embryos at the cotyledonary stage were counted. Fraction >450 μm gave *c.*105 and *c.*100 embryos for phase I and II, respectively. Cultures, which originated from phase III did not form any somatic embryos (Figure 2). PEM in phase II formed numerous somatic embryos after six weeks of culture, i.e. two weeks

earlier than PEM in phase I (Figure 3). Only PEM in phase II was selected for further experiments.

Cell suspensions of *G. cruciata* and *G. tibetica* differed in the percentage of five cell aggregate fractions studied (Table 1). In both species the most numerous were the aggregates from fraction 70-120 μm (54% in *G. cruciata,* and 36% in *G. tibetica).*

Growth parameters of the established cell suspensions were analysed during three consecutive years of culture at 12-month intervals. Both FW and DW decreased with extension of the culture age, but the FW/DW ratio increased (the highest value was obtained in the third year; the lowest, in the first year) (Table 2).

For both species flow cytometric DNA content in control plants, PEM and regenerants was studied (Table 3). PEM mitotic activity was evaluated by the comparison of the number of cells in the cell-cycle phases G_2 and G_1. The G_2/G_1 ratio was 6% and 18% for *G. cruciata* and *G. tibetica,* respectively.

Embryogenic capacities differed for both aggregate fractions of the species studied. In the presence of KIN, GA_3 and AS in the medium, cell suspension of *G. cruciata* was less productive than *G. tibetica*, and its fraction > 450 μm appeared to be superior. Fraction > 500 μm of *G. tibetica* gave at least four times more embryos than the same fraction of *G. cruciata* suspension.

The enrichment of the medium with AS resulted in the increased somatic embryo production in both species studied (Figures 4, 5). The statistical analysis revealed a significant effect of PGR concentration on embryo yield. Tables 4 and 5 show that the increased embryo production was correlated with the increase in KIN concentration up to 4.64 μmol. This concentration was found to be optimal for both species and fractions used. Its double increase did not significantly affect embryo production in *G. tibetica*, but it strongly reduced embryogenic competence in *G. cruciata* suspension.

Gibberellic acid played an important role in development and maturation of gentian somatic embryos. Although the most efficient embryo production was observed in *G. cruciata* in the absence of GA_3, many of embryos showed developmental disorders. Thus in *G. cruciata* GA_3 seemed to control the development of somatic embryos (Table 4). In both aggregate fractions of *G. tibetica,* GA_3 concentration did not affect the yield of somatic embryos (Table 5), however no embryos were obtained in the medium without GA_3 (Figure 6).

Table 1: Percentage of aggregate fractions in 3-year-old cell suspension of *G. cruciata* and *G. tibetica* (in 1.0 ml of suspension, after 5 days of subculture)

Aggregate fraction (μm)	*G. cruciata*	*G. tibetica*
70 –120	54 ± 0.59	36± 0.70
120-240	29 ± 0.54	30 ± 0.67
240-450	11 ± 0.37	14 ± 0.51
450-500	6 ± 0.27	17 ± 0.55
>500	0.6 ± 0.09	3 ± 0.24

Table 2: Ratio (R) of coefficient of fresh (FM) and dry (DM) mass of cell suspensions cultured in MS supplemented with Dic + NAA + BAP + AS

Age of culture	*Gentiana cruciata*			*Gentiana tibetica*		
	FM*	DM*	R	FM*	DM*	R
1-year-old	4.0	3.1	0.78	3.8	3.2	0.84
2-year-old	4.1	3.4	0.83	3.4	3.8	1.12
3-year-old	2.2	2.0	0.91	1.4	2.6	1.86

* ratio of final to initial cell mass

Table 3: 2C value describe by cytometic DNA (pg) content in control plant, 3-year-old PEM and regenerants of *G. cruciata* and *G. tibetica*. Mitotic activity of PEM - the ratio of cells in phase G_2 to phase G_1 (in bold)

Species	Control plants	PEM	Regenerants
G. cruciata	2.45 ± 0.13	1.5 ± 0.03 **(6%)**	2.77 ± 0.65
G. tibetica	2.51 ± 0.036	1.15 ± 0.35 **(18 %)**	2.74 ± 0.68

Table 4: Effect of GA_3 and kinetin (KIN) on the number of mature somatic embryos formed by 100 mg of *G. cruciata* embryogenic cells from suspension culture plated on agar medium supplemented with 434 µmol AS*. Data were collected between 6th - 10th week of the culture

KIN (µmol)	GA_3 (µmol)							
	0.0	1.49	2.89	Average SE_1=3.4	0.0	1.49	2.89	Average SE_1=3.26
	Fraction 240-450 µm a SE_3=5.965				Fraction >450 µm b SE_3=5.646			
0.0	257 a	189 c	146 de	197 a	103 cde	85 f	87 ef	92 b
2.32	139 ef	161 d	126 f	142 b	128 ab	82 f	81 f	97 b
4.64	231 b	202 c	150 de	194 a	135 a	105 cd	83 f	107 a
9.28	138 ef	129 f	146 de	137 b	117 bc	89 def	76 f	94 b
Average SE_2=2.98	191 a	170 b	142 c		120 a SE_2=2.82	90 b	82 c	

Table 5: Effect of GA_3 and kinetin (KIN) on the number of mature somatic embryos formed by 100 mg of *G. tibetica* embryogenic cells from suspension culture plated on agar medium supplemented with 434 µmol AS*. Data were collected between 6th - 10th week of the culture

KIN (µmol)	GA_3 (µmol)							
	0.0	1.49	2.89	Average SE_1=3.27	0.0	1.49	2.89	Average SE_1=4.059
	Fraction 240-450 µm *b* SE_3=5.659				Fraction >450 µm *a* SE_3=7.030			
0.0	0 *e*	201 *d*	213 *cd*	138 *c*	0 *d*	510 *c*	524 *c*	345 *c*
2.32	0 *e*	223 *bc*	230 *b*	151 *b*	0 *d*	581 *b*	592 *b*	391 *b*
4.64	0 *e*	291 *a*	288 *a*	193 *a*	0 *d*	634 *a*	629 *a*	421 *a*
9.28	0 *e*	288 *a*	290 *a*	193 *a*	0 *d*	620 *a*	630 *a*	417 *a*
Average SE_2=2.8	0 *b*	251 *a*	255 *a*		0 *b* SE_2=3.5	586 *a*	594 *a*	

* Data represent the average of 6 replicates (two independent experiments with 3 Petri plates). Effect of the aggregate fraction on embryo production was analysed by means of 1-factor variance analysis, effect of GA_3 and KIN and correlation between them was analysed by 2-factor analysis. SE_1 – standard error for kinetin; SE_2 – for GA_3; SE_3 – for aggregate fractions.

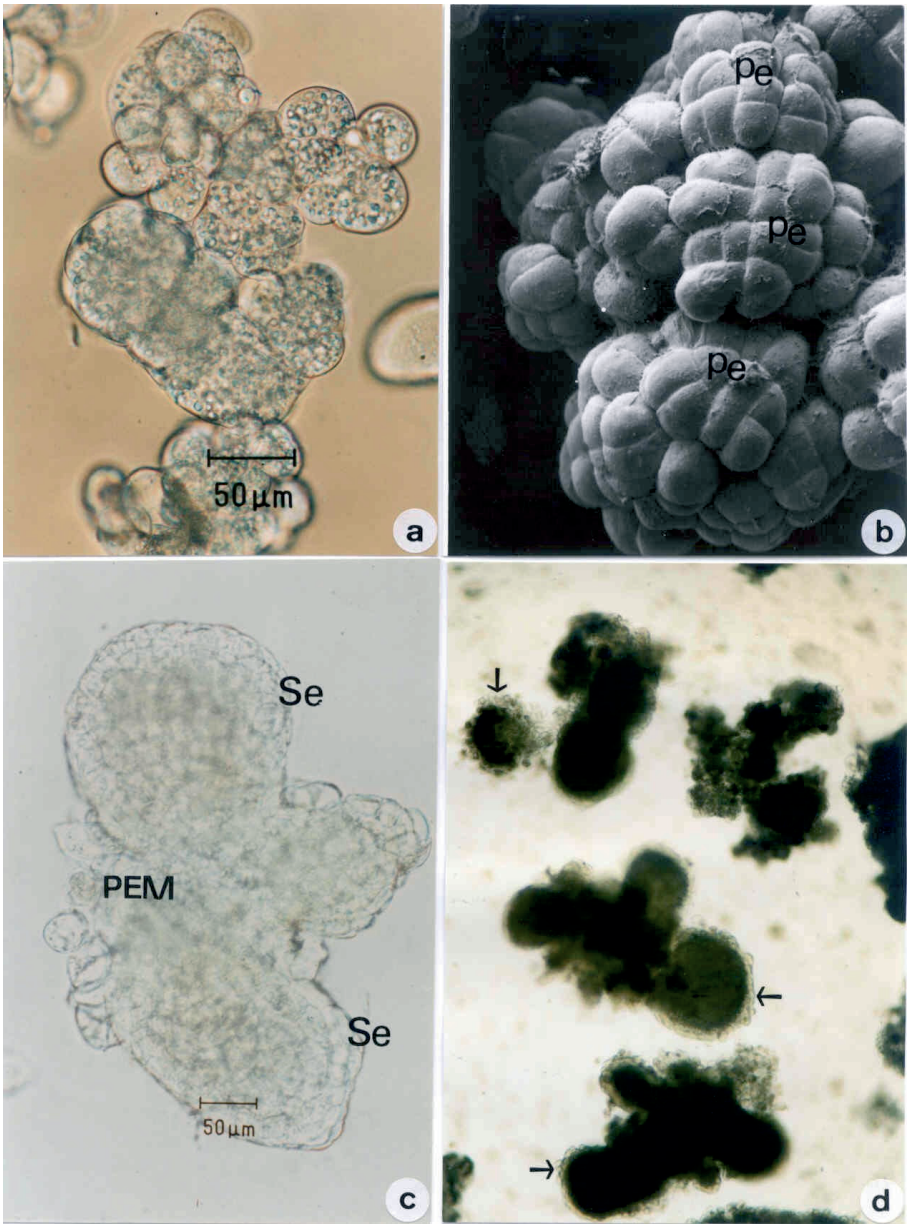

Figure 1: Phases of PEM development in *G. cruciata* suspension observed during 6 weeks of culture (MS+4.52 µmol Dic, 0,45 µmol NAA, 5.77 µmol BAP and 434 µmol AS) (intravital light microscope and SEM analysis):

a) cytoplasmatically rich embryogenic cells of PEM (Phase I)
b) scanning electonogram of PEM showing proembryos (SEM 720x)
c) PEM and globular embryos (Phase II) (SEM 600x)
d) Cell proliferation of epidermis of globular embryos (←) as the effect of extended auxin treatment of culture (Phase III).

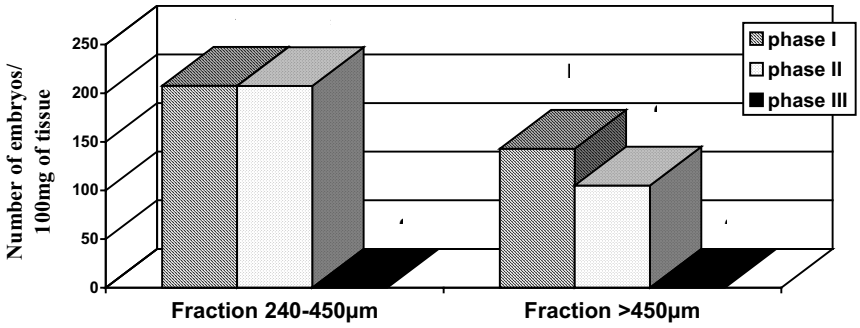

Figure 2: The effect of the cell growth phase on the total number of regenerated somatic embryos in *G. cruciata* (MS supplemented with 1.49 µmol GA_3 + 4.64 µmol KIN + 434 µmol AS) (SE=3.47).

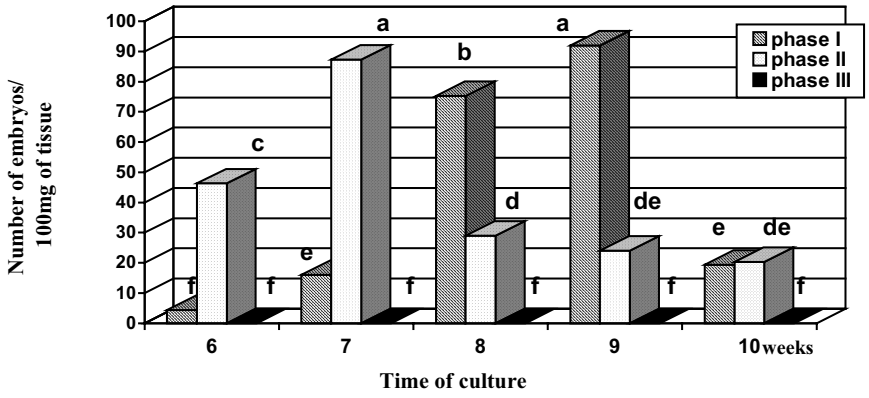

Figure 3: The effect of the cell growth phase on somatic embryo production in *G. cruciata* culture on MS supplemented with 1.49 µmol GA_3 + 4.64 µmol KIN + 434 µmol AS (SE = 3.134) in ten-week period (PEM fraction 240-450µmol).

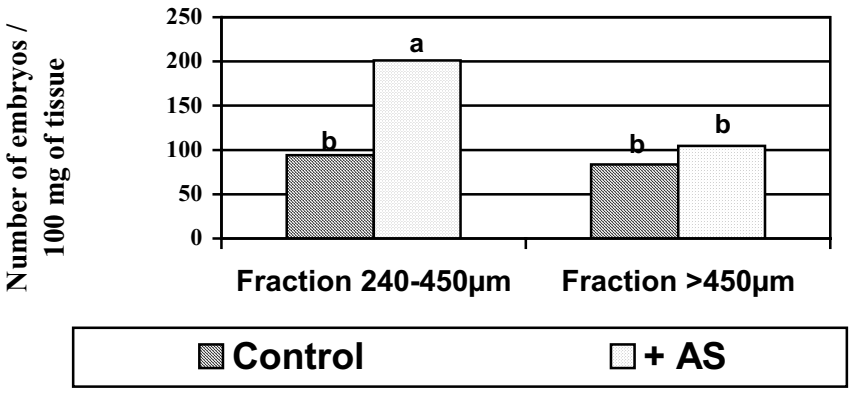

Figure 4: The effect of adenine sulphate (AS) on the total number of somatic embryos of *G. cruciata* on MS supplemented with 1.49 µmol GA_3 + 4.64 µmol KIN or without GA_3 and KIN (control), with (+AS) or without 434 µmol AS (SE=10.38).

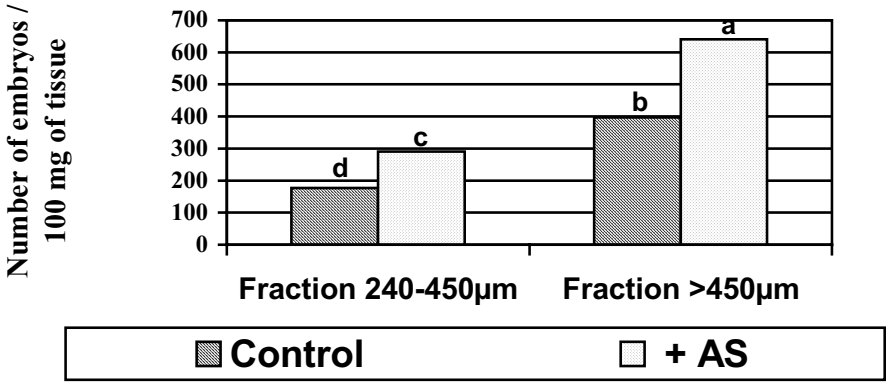

Figure 5: The effect of adenine sulphate (AS) on the total number of somatic embryos of *G. tibetica* on MS supplemented with 1.49 µmol GA_3 + 4.64 µmol KIN or without GA_3 and KIN (control), with (+AS) or without 434 µmol AS (SE=22.18).

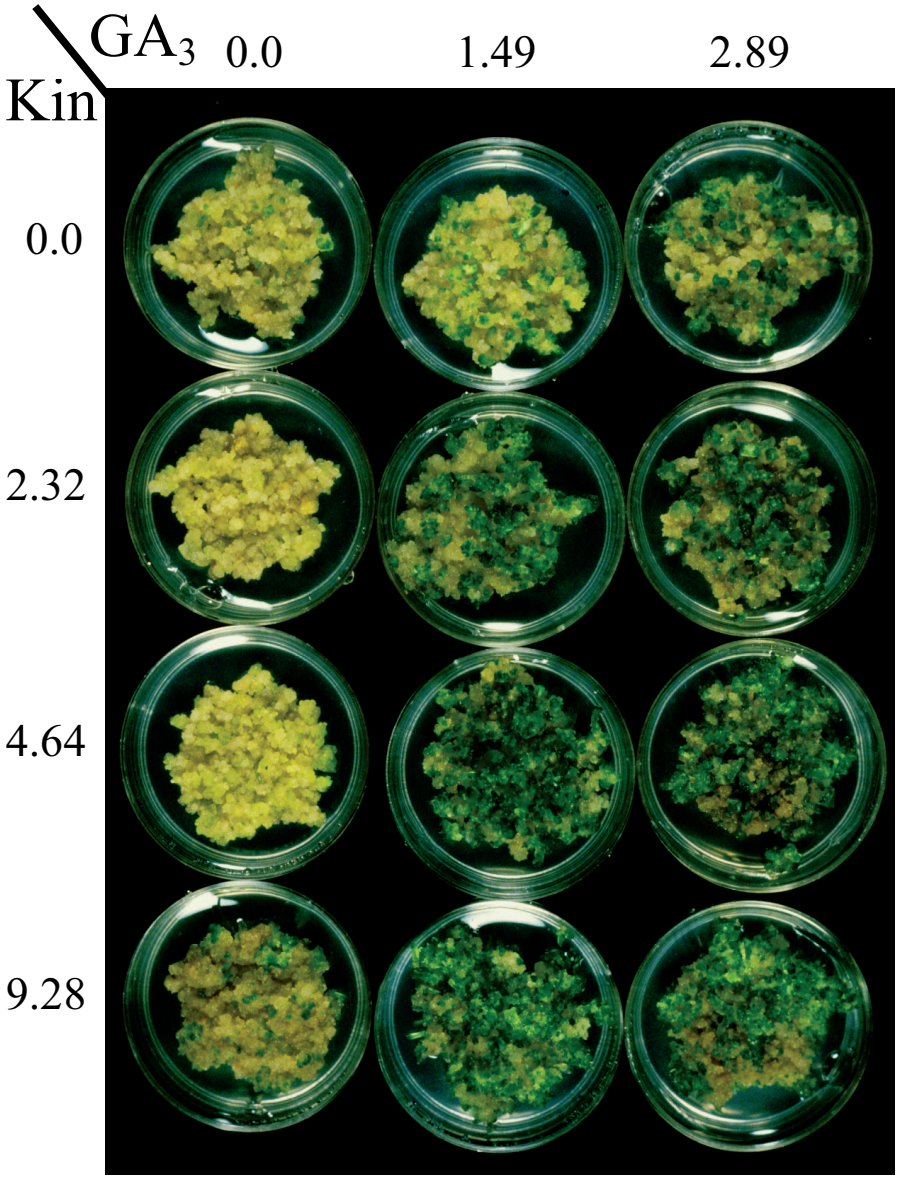

Figure 6: The development of PEM in *G. tibetica* on the solid medium supplemented with AS (434 µmol), GA_3 and KIN (different concentrations; µmol).

4. Discussion

Several differences have been found between the cell suspensions of *G. cruciata* and *G. tibetica*. Cell suspensions were described by the following parameters: percentage of five fractions of cell aggregate, ratio of fresh to dry mass, cytometric DNA content and cell cycle phase. Differences in the PEM development as well as in mitotic activity of cells have been observed. Flow cytometric analysis of nuclei DNA content revealed the differences between two suspensions studied. In both gentian cultures the flow cytometric analysis showed that the DNA content decreased almost by half in comparison to the leaf tissue of field-grown plants and regenerants (Table 3). The mitotic activity of suspensions could be expressed as the ratio of cells being in phases G_2 and G_1 of the cell cycle (Galbraith et al., 1983). In *G. tibetica* the G_2/G_1 ratio was threefold more than for *G. cruciata* cell suspension.

The most advanced development stage obtained in liquid cultures of *G. cruciata* and *G. tibetica* was the globular embryo. This stage was recognised in both aggregate fractions: 240-450 μm and >450 μm. These two fractions appeared to play an important role also in embryogenic cell suspension cultures of *Lisianthus russellianus* (*Gentianaceae*) (Ruffoni and Massabo 1996). In this species the highest yield of somatic embryos was obtained from the bigger fraction (>500μm) in the light. In darkness, however, the cell aggregates smaller than 200 μm also retained the embryogenic capacities. In cultures of *Exacum affine* (*Gentianaceae*) the cell fraction >100 μm was superior in comparison to smaller fractions and produced a large number of well-developed embryos (Ørnstrup et al., 1993). Cultures of *G. tibetica* appeared to be superior to *G. cruciata*. Fraction >450 μm in *G. tibetica* was almost three times more productive than the best fraction of 240-450 μm in *G. cruciata*.

The development of somatic embryos in gentian cultures requires implantation of cell suspensions onto an agar-gelled medium. It has been proved already that gibberellic acid plays an important role in morphogenesis of *Gentianaceae*. At a concentration of 1.99 μmol together with 13.86 μmol zeatin, 9.28 μmol BAP and 5.37 μmol NAA, it stimulated the growth of protocolonies of embryogenic calli of *Gentiana crassicaulis* (Meng et al., 1996). Additionally, GA_3 was required to stimulate shoot formation and their multiplication in *G. scabra* and *G. corymbifera* (Morgan et al., 1997; Yamada et al., 1991). In *G. punctata* GA_3 at a concentration of 0.289 - 2.89 μmol affected strongly the elongation of already existing and newly formed nodes (Vinterhalter and Vinterhalter, 1998). Also, GA_3 played an important role in rooting of the shoots of some gentians (Morgan et al., 1997).

Results presented here confirmed the important role of gibberellic acid in somatic embryogenesis in *Gentiana* species. Statistically significant differences were found in somatic embryo production in *G. cruciata* subjected to different concentrations of GA_3. In these cultures gibberellic acid controlled embryo development and conversion. In contrast to cultures of *G. cruciata*, PEM of *G. tibetica* did not formed any embryos on the medium lacking GA_3. The increase in the embryo production was correlated with the increase in GA_3 and KIN concentration. Similarly, in cultures of *G. pannonica,* PGRs used in the medium influenced embryo development (Mikuła et al., 2002a).

Cytokinins play a crucial role in plant morphogenesis. To induce shoot regeneration in such explants as leaf, shoot and root of *G. triflora* and *G. scabra* 90.8 µmol TDZ, was used (Hosokawa et al., 1996, Nakano et al., 1995). High concentration of BAP stimulated shoot differentiation in *G. scabra* (Takahata et al., 1995). Adenine sulphate supports the effect of other cytokinins used in the medium, but is not often used in tissue cultures of both mono - and dicotyledonous plants, as concentration usually does not exceed 27.1 µmol (Pradhudesai et al., 1972). In cultures of *Gentiana* species for callus initiation and bud formation, AS at concentrations of 434 and 217 µmol was previously used (Wesołowska et al., 1985). In our present study, cytokinins (KIN and AS) appeared to be indispensable for the long-term maintenance of embryogenic cell suspensions and for somatic embryo production. In both species of gentian studied, embryo production decreased when AS was not included in the medium. In cultures of *G. pannonica, AS at* 868 µmol in the medium showed the same inhibitory effect to when it was absent (Mikuła et al., 2002a).

5. Conclusions

Cultures in liquid and on agar-gelled medium were used in the experiment to characterise embryogenic cell cultures of two species of gentian. They differed in the response to culture conditions, however, they retained embryogenic capacities for a long time. The size of PEM aggregates had an influence on the regeneration abilities: for *G. cruciata,* the highest results were obtained for the size fraction 240-450 µm and for *G. tibetica,* fraction >450 µm was best. Kinetin and GA_3 promoted regular embryo development.

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