CYTOMORPHOLOGICAL STUDIES ON SOMATIC EMBRYOGENESIS OF *GENTIANA TIBETICA* (KING) AND *G. CRUCIATA* (L.)

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ABSTRACT

The process of plant regeneration via somatic embryogenesis of two gentianas, *Gentiana tibetica* and *G. cruciata* was described. For this purpose seedling explants were cultured on agar medium and later maintained in cell suspension. For callus initiation seedling explants like: cotyledons, hypocotyl and root were plated on a callus induction medium (CIM) composed of MS (1962), supplemented with 0.5 mg/l 2,4-D and 1.0 mg/l Kin. For the formation of cell suspension culture, embryogenic callus was transferred into liquid maintained medium (MM) composed of MS (1962), supplemented with 1.0 mg/l Dic + 0.1 mg/l NAA + 2.0 mg/l BAP + 80.0 mg/l SA. The conversion of somatic embryo into plantlets required a new medium (ECM) based on MS (1962) mineral salts, supplemented with 0.5 mg/l GA₃ + 1.0 mg/l Kin + 0.5 mg/l NAA. For cytomorphological studies of particular stages of embryogenesis, specimens were stained with dyes and reagents: 1. PAS reaction with leukofucsin, 2. Safranin + fast green, 3. Erlich's hematoxylin.

KEY WORDS: *Gentiana cruciata*, *G. tibetica*, somatic embryogenesis, cytomorphology, media, agar and suspension cultures.

INTRODUCTION

Species of *Gentiana* taxa are an object of interest because of their pharmaceutical value, i.e. production of biologically active substances. The most important are the secoiridoid glucosides (Skrzypczak et al. 1993). Among numerous gentiana species *G. tibetica* is of great significance. Recent progress in cell and tissue culture of gentianas (Skrzypczak-Pietraszek et al. 1993, Wesołowska et al. 1985, Mikuła and Rybczyński 1994, Lamproye et al. 1987, Sharma et al. 1993, Semeniuk and Griesbach 1987, Wesołowska et al. 1994, Bach et al. 1994) allows the use of biotechnological methods to utilize their naturally existing potentials.

Considering utilization of somatic hybridization in *Gentiana* genus and modern protection of endangered plant species to which Polish gentianas are included it was necessary to deve-

lop a long term and effective regeneration system initiated from a single cell or an embryogenic cell cluster.

Somatic embryogenesis as the way of plant regeneration allows to obtain a fully formed embryo originated from the somatic cell of any seedling or plant explant. Originated from this side embryos pass the same stages of that development like the zygotic one. The prerequisite of it is to develop particular culture conditions to ensure correct embryogenesis. In our cultures the mature embryo was characterized by numerous morphological disturbances, which usually occurred in agar medium cultures. It was observed that trumpet and monocotyledonary embryos most often developed in plantlets. In the case of disturbances consisting in the formation of a cauliflower-like and fused type of embryo no plant regeneration was observed (Goebel-Tourand et al. 1993). Using cytomorphological studies, we would like to learn more about the background of the observed disturbances which should help us to improve culture conditions to ensure a proper embryogenesis of the studied gentianas.

The aim of this paper is to show the cytomorphological changes of somatic embryogenesis, because of numerous disturbances observed in fully regenerated mature somatic embryo of the studied gentianas.

Abbreviations:

BAP – benzylaminopurine, SA – adenine sulfate, Kin – kinetin, Dic – dicamba, GA₃ – gibberelic acid, NAA – β -naphthaleneacetic acid, 2,4-D – 2,4-dichlorophenoxyacetic acid, MS – Murashige, Skoog 1962

Figs 1-6. Fig. 1. Section through 2 month-old embryogenic callus of *G. cruciata* used for cell suspension initiation. Notice: various types of cells with the tendency to form embryogenic cell centers (E). There are cells with thick cell wall (→) and numerous starch grains (→) inside. Fig. 2. Single cell originated 8-cell praembryo in cell suspension culture of *G. tibetica* (non-stained). Fig. 3. Numerous somatic globular embryos with epidermis derived from cell suspension of *G. tibetica* (non-stained). Fig. 4. Correctly formed globular somatic embryo of *G. cruciata* with distinct suspensor (S) and praepidermis (E) surrounded by the praembryogenic mass (PEM) (Erlich's hematoxylin). Fig. 5. Globular (G) and beginning of heart stage embryo (H) on embryogenic callus (EC) of *G. cruciata*. Notice starch grains (→) in embryo cells and deeply located suspensor (S) in callus with the lack of bundle of vascels junction (PAS + leucofucsin). Fig. 6. Typical heart stage embryo of *G. cruciata* observed in cell suspension culture (safranin + fast green).

MATERIAL AND METHODS

Plant Material

Seeds germination and seedlings culture of *Gentiana tibetica* (King) and *G. cruciata* (L.) follow the earlier described methods (Wesołowska et al. 1985). The seedling explants like: cotyledons, hypocotyls and roots originated from 42 day-old seedling were planted on CIM for callus induction. After 5 months of culture the callus tissue was transferred into liquid MM. For somatic embryogenesis completion the medium was changed to EgCM. The next stages of the culture required the following media: ECM and RMM

Media used:

Consecutive stages of gentiana cultures required the use of Murashige and Skoog's medium (1964) supplemented with various plant growth hormones:

Callus Induction Medium (CIM) –

MS (1964) + 0.5 mg/l 2,4-D + 1.0 mg/l Kin (Skrzypczak et al. 1985).

Maintaining Medium (MM) –

MS (1964) + 1.0 mg/l Dic + 0.1mg/l NAA + 2.0 mg/l BAP + 80.0 mg/l SA.

Embryogenesis Completing Medium (EgCM) –

MS (1964) + 0.24 mg/l GA₃ + 0.24 mg/l Kin + 100 mg/l SA + 500 mg/l casein hydrolizate.

Embryo Conversion Medium (ECM) –

MS (1964) + 0.5 mg/l GA₃ + 1.0 mg/l Kin + 0.5 mg/l NAA.

Regenerant Maintaining Medium (RMM) –

half concentration of MS (1964) salt with all vitamins.

Cytomorphological examination

Cytological studies were carried out using three various types of staining. Culture derived samples of callus tissue (originated from agar medium culture), praembrogenic mass and somatic embryos in various stages of development (originated from cell suspension culture) were fixed in formaldehyde: acetic acid: 70% alcohol, 1:1:3 (FAA) solution for 24 h and then stored in 70% ethanol. After dehydration and wax embedding, 5-10 µm specimens were cut. For starch localization the PAS reaction with leucofuchsin was employed. The next two stainings: safranin + fast green and Erlich's hematoxylin helped to describe embryogenic and non-embryogenic character of cultures. Additionally, some analyses were based on Nomarsky's contrast and non-stained squash *in vivo* specimens. The analyses were made on a light microscope NA-VOX with UV system of Olympus. Colour pictures were taken using Fuji Color Films 100.

RESULTS AND DISCUSSION

After five weeks of culture on CIM medium explants responded by formation of a callus dedifferentiation usually of the surface on the cotyledon of zygotic embryos. In the case of the others whole isolated organs dedifferentiated callus tissue. Due to culture conditions two types of callus tissue with varying morphogenic potential were obtained. Although differences in response of the studied explants and species were noted, one of the obtained calli was of embryogenic character (Mikuła and Rybczyński 1994). Using two systems of culture of the embryogenic tissue, i.e. agar and liquid medium, it was possible to observe the development of consecutive stages of somatic embryos and their conversion in to plantlets.

The transfer of embryogenic callus originated from the callus induction medium (CIM) (Fig. 1) to the liquid medium initiated cell suspension culture. The most important prerequisite of development of its embryogenic character is to acquire a cell meristematic activity in new aquatic conditions of the maintaining medium (MM). Later on the formation of somatic embryos through praembryogenic mass (PEM) (Fig. 3) or directly from single totipotent cells (Fig. 2), which undergo differentiation, initiate meristematic activity (Williams and Maheswaran 1986). The totipotent cells are usually smaller than the other ones and have a large nucleus with nucleolus and dense cytoplasm. In our studies the use of Erlich's-hematoxylin staining allows to show groups of cells, which stronger absorbed dyes than others and carried an embryogenic character (Fig. 4). The cytoplasm of these cells has numerous starch grains, proved by the staining of PAS reaction and leucofuchsin (Fig. 5). Here, distinct meristematic centres could be observed, too. They included a group of some or a several surrounded by the thin cell wall, separated from other cells by thick and strongly stained wall (Botti and Vasil 1984). As the result of subsequent cell divisions somatic praembryos were formed (Halperin 1966). In the beginning, the praembryos consisted of non-differentiated cells carrying meristematic character cells. Gradually, as praepidermis was formed, external cells tightly adhered to each other and elongate. The globular stage of embryo is characterized by well developed epidermis and well shaped bipolarity of the axis. In the basal part the suspensor cells help globular embryo to be in touch with the mother tissue (Fig. 4). Intensive cell divisions resulted in overgrowth of the embryo, in its plumular part (hard stage) (Fig. 6), and its next differentiation. The gradual differentiation of praeambium and praparenchyma cells appeared. Figure 5 presents characteristically elongated cells, closely adhering to each other, which have been formed from radicular towards plumular pole, without starch, forming praeambium. They are surrounded by cells presenting typical arrangement of parenchyma cells which show irregular shape, common size and numerous storage substances.

The transfer of not completely formed embryos (Fig. 7) from darkness to light conditions showed their early functional division into three naturally existing parts, i.e. cotyledons, hypocotyl and root, if the presence of organells in their epidermal cells was taken into consideration. In cotyledon epidermal cells possessing chloroplasts which lose the green colour towards root, where only leucoplasts were recognized, finally lacking of plastids in the suspensor cells.

Further development required the change of the medium to EgCM, which was supplemented with GA₃. In such conditions embryos reached maturity. Figure 8 gives examples of the embryo. Although normally shaped embryos with well formed cotyledons were predominantly found, numerous developmental disturbances were recognized in our cultures. It seems that the medium used only partly satisfies hormonal requirements of correct embryogenesis.

Most often trumpet embryos could be found in cultures of both the studied *Gentiana* species. This type of disturbances and the one like fused embryo (Fig. 9) have been very often described in other somatic embryogenesis systems (Amirato 1987, Goebel-Tourand et al. 1993). Looking closely to this problem, in our cultures the hypocotyl elongation and limited formation, and differentiation of the cotyledons were recognized particularly in the case of embryo originated from cell suspension. Additionally the influence of darkness in which the suspension cultures were carried on is not insignificant.

Figs 7-9. Fig. 7. Somatic embryo of *G. tibetica* and their parts after light exposition (nonstained). Embryos derived from cell suspension culture carried in the dark. These parts are easily recognized according to number of type and number of plastids present in epidermal cells. A. general view, B. cotyledon – like part with dense chloroplasts, C. hypocotyl – like part with rare chloroplasts, D. radicular – like part with leucoplasts, E. suspensor – like part.

Fig. 8. Most often selected embryos originated from agar medium cultures.

Fig. 9. Fused embryo selected from *G. tibetica* culture with emerging first leaf. C – cotyledon, L – leaf, R – root.

As in the case of the mentioned above embryo disturbances, the disorder of embryogenesis occurred after reaching the heart stage by the embryo. The fused forms of embryos originated most probably from very early stages of embryogenesis or even from praembryogenic mass. Twin embryos recognized in microspor cultures of wheat could confirm the single cell origin (Rybczyński et al. 1991). If the observed disturbances are not connected with the plumul and do not damage the apical meristem and leaf primordia differentiation, the embryo conversion to plantlets can happen on ECM (embryo conversion medium). However, the time required for conversion was longer than in regularly formed embryos. The transfer and later culture of regenerants on RMM helped to compensate the observed developmental differences, which were the results of earlier described morphological disturbances. The morphological disturbances of further development of embryos can contribute to somaclonal variation which is an undesirable feature while material is cloned for preservation of endangered plant species.

The presented system of *Gentiana tibetica* and *G. cruciata* plant regeneration via somatic embryogenesis in cell suspension culture, even though it includes a few steps of culture, did not prevent numerous morphological disturbances. The figures showed here prove that the proces of somatic embryogenesis happend correctly up to heart stage and, maybe even, to torpedo stage. However, the number of converted embryos to plantlets is relatively low, considering the huge number of obtained somatic embryos in this regeneration system. We are convinced that this analysis will help us to modify the whole culture system, which should finally give a high percentage of proper somatic embryos.

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CYTOMORFOLOGICZNE BADANIA NAD SOMATYCZNĄ EMBRIOGENEZĄ *GENTIANA TIBETICA* (KING) I *G. CRUCIATA* (L.)

STRESZCZENIE

W pracy opisano proces powstawania roślin *Gentiana cruciata* L. i *G. tibetica* King drogą somatycznej embriogenezy. W kulturze agarowej stwierdzono występowanie w tkance kalusowej centrów komórek totipotnych, z których następnie powstawały zarodki. W pożywce płynnej zaobserwowano dwie drogi różnicowania się zarodków: z PEM-u (masy praembryogennej) i z pojedynczych komórek. Różnicowanie się i kolejne stadia zarodków somatycznych przedstawiono w oparciu o preparaty parafinowe barwione różnymi metodami: reakcja PAS i leukofuksyna, safranina i zieleń trwała, hematoksylina Erlicha.

SŁOWA KLUCZOWE: *Gentiana*, kultury zawieszinowe, preparaty cytologiczne, somatyczna embriogeneza.