ULTRASTRUCTURAL ANALYSIS OF INITIAL STAGES OF DEDIFFERENTIATION OF ROOT EXPLANTS OF *GENTIANA CRUCIATA* SEEDLINGS

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ABSTRACT

The studies were carried out on isolated roots of 10-day old seedlings of *Gentiana cruciata*, which were placed and cultured on induction medium of Murashige and Skoog (1962) supplemented with 1.0 mg/dm³ dicamba + 0.1 mg/dm³ NAA + 2.00 mg/dm³ BAP + 80.0 mg/dm³ adenine sulphate. Changes in explants from the 3rd to the 11th day of culture with the help of light and electron microscope were observed. Observations showed gradual dedifferentiation of root tissues, which was seen earliest in cortex at the proximal end of the explant and shifted gradually inwards the root and towards distal parts of its elongation zone. The most intensive callus formation appeared at cut surface of explant, where proliferation of cells in both cortex and axial cylinder was recognised. In the distal part of the elongation zone, cell divisions occurred only in endoderm and in axial cylinder. The meristematic part of the root was inactive. Finally, the following areas were distinguished in the explant: (I) an area of intensive cell divisions, i.e., the elongation zone; (II) an area of cell dispersion; and (III) the inactive meristem.

The ultrastructure brought evidences of cell reorganisation as the meaning of cell readiness to the division. Observations showed an increased activity of mitochondria and Golgi structures, thickening of walls and disappearance of plasmodesmal connections. Amyloplasts and lipid bodies in tissues in which they had been scarce or had not appeared before founding. Intensively dividing cells showed features of meristematic cells. They had dense cytoplasm with numerous organelles, large centrally located nuclei, and „nucleolar vacuoles” inside nucleoli. Cortex-derived callus formed aggregates. Both pericycle and endoderm produced callus of characteristic dense structure and regular type of divisions.

KEY WORDS: seedling root of *Gentiana cruciata* L., dedifferentiation, ultrastructure, callus development.

INTRODUCTION

Species of *Gentiana* genus are plants of horticultural and medicinal value. Most of them are protected by law. They are difficult to reproduce both vegetatively and generatively. For this reason, more and more often their culture in vitro is contemplated in studies. So far, 12 species have been introduced into in vitro culture and regenerated mostly via organogenesis (Mikuła and Rybczyński 1999). The process of somatic embryogenesis has so far been described only for six species. The ultrastructural analysis was done only for four of them. In *G. cruciata*, *G. pannonica* and *G. tibetica*, a thorough analysis of PEM was carried out and differentiation of somatic embryos in cell suspension culture were shown (Mikuła et al. 2001). In *G. punctata*, ultrastructural studies were carried out for initial stages of dedifferentiation of zygotic embryos in agar medium (Mikuła et al. 2000).

Investigations concerning morphogenetic competence of particular explants of *Gentiana* plant showed, apart from species limitations (Momčilović et al. 1997; Mikuła and Rybczyński 2001), also a significant variation of response of particular types of explants within a single species (Hosokawa et al. 1996). After two weeks of culture cotyledons and hypocotyls of *G. cruciata*, *G. pannonica* and *G. tibetica* and roots of seedlings of *G. pannonica* produced callus, which after the next three weeks of culture appeared embryogenic and produced somatic embryos at different stages of development (Mikuła et al. 1996; Mikuła and Rybczyński 2001). Roots of *G. pannonica* showed the poorest ability to form embryogenic callus, but cultures originated from *G. cruciata* and *G. tibetica* were not competent to produce embryos (Mikuła and Rybczyński 2001).

Different morphogenic abilities of particular seedling explants inclined us to study the structure and ultrastructure of cells in the process of dedifferentiation of tissues and

proliferation of callus in the presence of induction medium in the first days of culture. The present work concerns the ultrastructural analysis of initial stages of dedifferentiation of root explants of *G. cruciata* and constitutes the first stage of observation of response of the seedling explant to culture conditions.

The aim of the studies was to evidence changes which accompany formation of callus tissue in root explants of *Gentiana cruciata* and their ultrastructural determination.

MATERIAL AND METHODS

Plant material

The objective of our studies were root explants of 10-day old seedlings of *Gentiana cruciata* (Fig. 1a). Seeds of gentians were disinfected with 10% Domestos by 20 minutes and rinsed three times in sterile distilled water. Disinfected seeds germinated in light, on 1% agar only. Root explants were cut off below root-shoot junction (see Fig. 1a) and placed on MS medium supplemented with 1.0 mg/dm³ of dicamba, 0.1 mg/dm³ NAA, 2.0 mg/dm³ BAP and 80.0 mg/dm³ adenine sulphate. The medium was adjusted to pH=5.6 and solidified with 0.8% agar (Difco Bactoagar). Culture were initiated and maintained in 6 cm sterile plastic Petri dishes. The proximal end of explant describes the part located nearer to the shoot-root junction, the distal one located down in direction to root meristem.

Cultures were maintained in a culture chamber, with a photoperiod 16/8 day/night at 22±1°C. For microscope analyses, explants were collected after 3, 4, 5, 6, 8 and 11 days of culture. Control explants were excised from 10-day old seedlings growing in 1% agar.

Microscopic preparations

Samples of material were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 2 hours, followed by additional fixation in 2% OsO₄ for 2 hours at 4°C. They were then dehydrated using a graded series of ethanol and propylene oxide and embedded in a mixture of Epon and Spurr. Semi-thin (1-2 µm) and ultrathin (80 nm) sections were cut off with an LKB ultramicrotome (Sweden). Semi-thin sections were stained with 0.1% toluidine blue in 1% borax and they were analysed in a light microscope Navox-Olympus. Ultrathin sections were contrasted in water solution of uranyl acetate (30 min.) and lead citrate (30 min.) according to Reynolds (1963). The sections were investigated in transmission microscope (JEOL JEM 100C). Electron micrographs were taken on 60×90 mm negative film (TN-12 Foton).

RESULTS

Microscopic analysis of a control explant, excised root (Fig. 1a), consists of typical two zones: meristematic (Me) and elongation (El). Fig. 1b shows a characteristic structure of cells in that organ: epidermis, cortex with endoderm, and axial cylinder with a pericycle. In a cross section, the different appearance of particular tissues and one-layer structure of endoderm and pericycle (Fig. 1c) is especially distinct. Radial structures of inner layers of cortex and scarce intracellular spaces are also symptomatic. In the me-

ristematic zone (Fig. 1d), dermatogen and periblem cells were transversely flattened, closely adjoining one another, and plerome cells were oblong. All cells of that zone had typical ultrastructure of meristematic cells. They contained dense cytoplasm with numerous small vacuoles, amyloplasts and proplastids and large centrally located nuclei. In the elongation zone, cells of both epidermis and axial cylinder were of oblong shape and contained large vacuoles. Cytoplasm was often found in them as a thick layer adjacent to the wall, and the nucleus was pushed towards peripherals of the cell (Fig. 1e). Amyloplasts were observed mainly in two inner layers of cortex cells (including endoderm) and in the pericycle (Fig. 1f), but only in axial cylinder numerous proplastids without starch were found (Fig. 1g).

Observation and comparison of response of particular tissues of explants in culture conditions in subsequent days of culture (from 3rd to 11th) with the control allowed a thorough study of the occurring changes. After 3 days of culture (Fig. 2), in the proximal part of the elongation zone, decay of epidermis cells, enlargement of intracellular spaces and disturbances in the normal, orderly, radial structure of cortex cells were observed (Fig. 2a). It has been found, that just under the cut surface of the explant the cortex cells underwent the earliest dedifferentiation. They acquired features of meristematic cells and passed through subsequent divisions (Fig. 2b). In those cells found was an increased activity of Golgi structures, intensively producing dictyosomal vesicles, which penetrated through plasma-lemma into cell walls (Fig. 2c). Meanwhile, in the thick middle lamella, electron-lucent spaces appeared, indicating the onset of pectin hydrolysis process, leading to separation of cells (Fig. 2d). In thickening walls, diminution of plasmodesmal junctions occurred, although they were numerous in walls of newly-divided cells (Fig. 2e). Apart from the cortex, also pericycle (Fig. 2a) and endoderm (Fig. 2e, 2f) cells underwent divisions. While cortex cells at the cut surface acquired meristematic features and passed through their first divisions, cells below that place preserved their elongation (Fig. 2g). Onset of dedifferentiation of those cells may be indicated by the appearance of numerous amyloplasts containing large number of starch grains.

The analysis of explants after five days of culture (Fig. 3) showed many divided cells at the explant cut surface (Fig. 3a) and in its elongation zone below that surface (Fig. 3b). As a result of subsequent divisions of single cells isolated from one another, with a thick wall of cortex cells, cell aggregates were found (Fig. 3c). They had a dense structure and divided in an orderly way. Their cytoplasm contained numerous, very large amyloplasts with starch grains, vacuoles of various size, and large, centrally located, active nuclei. Among particular aggregates, large intracellular spaces were seen, and the multicellular aggregates themselves were surrounded by a thick wall. Distinct isolation of those structures from surrounding tissue, their shape and sequence of cell divisions were typical for forming proembryos (Fig. 3c). Simultaneously, to divisions in the cortex leading to formation of the aggregates, periclinal cell divisions occurred in the endoderm (Figs 3b, d, e) and pericycle (Fig. 3b, f) cells, which led to their multi-layer structure. The intensity of cell divisions depended on the distance from the explant section. While the endoderm consisted of 3 layers of cells in the vicinity of the root section (i.e., in the proximal part of elongation zone – ELB), it

Fig. 1. Structure and ultrastructure of the root of 10-day old seedling of *Gentiana cruciata* – control.
a) A general appearance of a 10-day old seedling (asterisk – place of cut), (dissecting microscope picture).
b) Longitudinal section through an explant of a seedling root (semi-thin specimen)
c) Typical structure of tissues in the proximal part of the elongation zone of the root – cross section
d) Ultrastructure of dermatogen and periblem cells in the meristematic zone of the root – rich cytoplasm (arrow – proplastids.), large centrally located nuclei (N) (longitudinal section) (1200×)
e) Ultrastructure of oblong cells of the elongation zone – large vacuoles (V), amyloplasts (Am) visible in the cortex layer adjacent to the endoderm (longitudinal section) (1200×).
f) Numerous amyloplasts (Am) in the endoderm (En) and pericycle (P) (cross section) (1300×).
g) Proplastids (pPl) in the cells of axial cylinder (magnification 8000×).
Symbols for Figs 1-5: C – cotyledon; H – hypocotyl; R – root; Me – meristematic zone; EL – elongation zone; Am – amyloplasts; N – nuclei; V – vacuoles; Ep – epidermis; En – endoderm; P – pericycle; Se – subepidermis; C – primary cortex; Vc – axial cylinder.

Fig. 2. Structure and ultrastructure of the root after three days of culture in the initial medium

- a) Cross section through the root in proximal part of the elongation zone in the vicinity of the place of cut – note the irregularly divided cells of pericycle (P), endoderm (En) and cortex (C), enlarged intercellular spaces (IS), folded walls and decayed epidermis cells (EP) (semi-thin specimen)
- b) Ultrastructure of cortex cells after the first division – numerous organelles visible: amyloplasts (Am), proplastids (pPl) (cell wall – Cw; nucleus – N; vacuole – V) (cross section) (2300×).
- c) Numerous active Golgi structures (GA) intensively producing dictyosomes (arrows) included into the cell wall (Cw) (mitochondria – M) (14000×).
- d) Irregularly thickened cell wall (Cw) with a thick layer of pectins and electron-lucent vesicles visible within it (8000×).
- e) Thin walls with numerous plasmodesmata (arrows) in newly-divided cells (6000×).
- f) Metaphase in a strongly vacuolated endoderm cell (cross section) (1200×).
- g) Longitudinal section through the root in the elongation zone far from the place of cut. Amyloplasts (*) visible in all cortex cells (longitudinal section) (1400×).

Fig. 3. Changes in the root after 5 days of culture

- a) Longitudinal section through the proximal part of the elongation zone of the seedling root explant – numerous divided cells visible at the border of the cut (semi-thin specimen).
- b) Cross section through the proximal part of the elongation zone below the place of cut – note multicellular aggregates originated from divisions of the cortex cells (C), multi-layered endoderm (En) and two-layered pericycle (P); lack of divisions in the subepidermis (Se) (semi-thin specimen).
- c) Ultrastructural picture of aggregates originated from single cortex cells (the section was made below the place of cut of the explant). Note large amyloplasts (Am) abundantly filled with starch, numerous vacuoles of different size (V), large flattened nuclei (N), large intercellular spaces (IS) (cross section) (1400x);
- d) The two-layered endoderm of the elongation zone of the root far from the place of cut (cross section) (semi-thin specimen)
- e) Ultrastructure of divided meristematic endoderm cells – large, centrally located nuclei (N), numerous amyloplasts (Am), Golgi structures (GA) (cross section) (1400x).

Fig. 3. Cont.

- f) Ultrastructure of a divided pericycle cell (vacuole – V; axial cylinder – Vc; endoderm – En) (cross section) (1800x).
- g) Granular structure of nucleolus and presence of „nucleolar vacuoles” (VNu) in meristematically active cells (10 000x);
- h) Ultrastructure of axial cylinder cells: numerous proplastids (pPl) in the axial cylinder cells (10 000x);
- i) Axial cylinder cell preserved typical oblong shape, with lengthened nuclei (N) and, often, with double nucleoli (Nu). Appearance of numerous amyloplasts (Am) containing starch (1700x);
- j) Presence of starch in amyloplasts (Am) and lipid bodies (Lb) in axial cylinder cells (8000x);
- k) Numerous Golgi structures (GA) and rough endoplasmic reticulum (rER) in the same cells (8000x).

had 1 or 2 layers below that place (Fig. 3d). Endoderm cells, which were previously strongly vacuolised (Figs. 1f, 2f), changed their character to the typically meristematic and they contained cytoplasm abundant in cell organelles (Fig. 3e). In many active cells of the meristematic type, the nuclei possessed dispersed chromatin and actively appearing nuclei with a distinct granular and fibrillar part and vacuole inside (Fig. 3g). Changes in the axial cylinder were also observed. Ultrastructure of its cells revealed, apart from proplastids (Fig. 3h), amyloplasts, which were absent in the control (Fig. 3i), and lipid bodies (Fig. 3j). Numerous active mitochondria and rough endoplasmic reticulum (Fig. 3k) were observed in the cytoplasm. Cells of the central cylinder persisted, however, their initial oblong shape, and had not divided by that time.

After 8 days of culture (Fig. 4), the enlargement of cell aggregates originated from the cortex and further divisions of endoderm and pericycle were observed. Cell divisions were found to move centripetally towards the axial cylinder (Figs. 4a, b).

After 11 days of culture, the explants showed a structural differentiation allowing to distinguish the following zones (Fig. 5a):

1. Zone of intensive cell divisions – located in the proximal end of the elongation zone of the explant (ELB). In that zone, divisions were initiated earliest just below the isolation surface, starting from cortex and pericycle cells, and then in the axial cylinder. They moved inside and in apical direction, thus forming a large area of callus;

2. Zone of cell dispersion – containing the distal part of the elongation zone (ELA). Cortex cells were dispersed and gradually destructed, and cell divisions occurred in the endoderm, pericycle and the axial cylinder;

3. Zone of inactive meristem (Me) – containing the meristematic part of the root, the function of which was inhibited and no structural changes were observed. Its cells were meristematically inactive, strongly basophil, small and of uniform shape, and they lost their typical meristematic function of cell divisions.

Cortex derived callus had, at the cross section, a loose and distinctly aggregate structure, whereas, at the longitudinal section, both whole aggregates and many cells inside them were of oblong shape (Fig. 4a). Unlike in that tissue, dividing cells of the axial cylinder, in its whole elongation zone, preserved their dense structure and were closely adjacent one to another. Their divisions at the proximal end of the explant were more numerous and of various orientation (Fig. 5b). On the 11th day of culture, in the vicinity of ELA and ELB zones, a 3-4-layer pericycle was observed, with cells dividing periclinally and anticlinally, and of radial cell structure (Fig. 5c). In large cortex aggregates a secondary dedifferentiation, shifting towards the center, was observed (Fig. 5d). The aggregates included various types of cells. Outer cells of the aggregates were meristematically active and had a dense cytoplasm with numerous organelles. Cytoplasm of inner cells of the aggregates was located adjacent to the walls, and the center of cells was occupied by huge vacuoles (Fig. 5e).

The studies of the present work allowed observation of gradual dedifferentiation of root tissues, which was seen at the earliest in the cortex at proximal end of explant and shifted towards inside of the root, overwhelming simulta-

neously the lower parts of its elongation zone. The most intensive formation of callus was found at the section of the root, where proliferation of cells occurred both in the cortex and the axial cylinder. In the distal part of the elongation zone, cell divisions were only found in the endoderm and the axial cylinder, while the meristematic part of the root did not mitotically respond and got inactive (Fig. 5f).

DISCUSSION

The explant isolation disturbs the naturally occurring balance of life events. In induction of callus tissue, similarly as in formation of somatic embryos, apart from species-related limits and the culture itself, the type, developmental stage and physiological status of the explant are of importance (Street 1978; Williams and Maheswaran 1986). In dicotyledons for culture initiation various explants can be used, but in many cases not all their tissues participate in callus formation. Callus tissue could be formed in the whole explant or in its part. Sometimes it appears only at surface of the section. In present studies on root explants of *G. cruciata* seedling, the earliest and most abundant formation of callus tissue was observed at the cut surface and in the proximal part of the elongation zone of the root. This phenomenon is a typical response of wounded tissue. The damage causes increase of activity of many enzymes and changes of endogenic plant growth regulators concentration, which is important for cicatrization of the wound. On the other hand, also changes in activity of ion channels regulating intensity and composition of ion flow, and leading to polarisation of cells. The existing micropolarisation in explants causes induction of callus tissue, which appears in places of the highest gradient of electric potential. This usually pertains to parts of tissues containing the youngest or damaged cells (Filek et al. 1994). Initially, mainly at the cut surface, due to activation of ion transport, cell elongation or formation of callus could be seen. Formation of callus tissue at the wounding site has also been observed in hypocotyl and cotyledon explants of *G. cruciata* and *G. tibetica* and at cotyledons of *G. pannonica* (Mikuła and Ryczyński 2001).

It was shown that the first stage of dedifferentiation of cortex cells was their isolation, manifesting itself by thickening of walls, disappearance of plasmodesms, enlargement of intracellular spaces and loosening of middle lamellae. Changes in chemism of cell walls (Nishitani et al. 1979) and their loosening allowing subsequent growth of cells (Masuda 1977) are due to presence of growth substances, and particularly high level of auxins in comparison with cytokinins. Separation of cells was probably an effect of increased synthesis and secretion of hydrolytic enzymes, causing loosening of pectins of middle lamellae (Fransz and Schel 1991) responsible for contents of cells. The disappearance of plasmodesmal junctions between cells resulted in change in type of transport of nutritive substances from symplastic to apoplastic, i.e., by diffusion (Fransz and Schel 1991; Verdeil et al. 2001). Isolation of cells allows them to escape from gradient of growth substances and to follow the path of somatic embryogenesis, either direct (Konar et al. 1972; Trigiano et al. 1989) or indirect – through callus tissue (Franz and Schel 1991; Mikuła et al. 2001).

Fig. 4. Changes in the root after 8 days of culture

a) Longitudinal section through the root explant in the proximal part of the elongation zone (ELB) – note large, lengthened cell aggregates of the cortical (C) origin and centrally located cells with symmetric divisions, of endodermal (En) and pericycle (P) origin (semi-thin preparation).
b) Structure of the root explant at a longitudinal section in the proximal part of the elongation zone – note multi-cellular aggregations of cortical (C) origin, pericycle (P) cells divided into 3 – 4 layers, and irregular divisions of axial cylinder cells (semi-thin preparation).

Fig. 5.

Fig. 5. Changes in the root after 11 days of culture

a) Longitudinal section through the callus-producing root explant, with the following zones are distinguished: the zone of intensive divisions, located in the proximal part of the elongation zone of the root (ELB); dispersion zone, comprising the distal part of the elongation zone of the root (ELA); the zone of inactive meristem, comprising the meristematic part of the root (Me). (the arrow indicates the place of the cross section for Fig. 5c; the dashed line shows the border between the zones).

Fig. 5. Cont.
b) Structure of the root explant at a cross section in the place of cut – note separate cell aggregates of cortical origin, of different size (1, 2, 3, 4), and numerous, irregularly divided cells of the axial cylinder (Vc) (semi-thin specimen)
c) Multi-layered pericycle (P) originated as a result of periclinal divisions of the one-layered initial tissue (cross section in the place indicated in Fig. 5a).
d) Cell vacuolation (V) progressing towards the middle of explant (cross section) (semi-thin specimen).
e) Ultrastructure of cell aggregate – note very expanded amyloplasts (Am) and strongly vacuolated (V) cells inside, thick cell wall (Cw) and large intercellular spaces (IS) (cross section) (1000x).
f) Macroscopic specimen of activity of growth of callus tissue at the whole length of the elongation zone of the root (EL). Note intensified formation of callus in the place of cut of the explant and its lack in the meristematic zone (Me). (length: 2 cm)

The cascade of observed changes occurring in cells of the root explant, discussed herein, were similar to modifications appearing during acquisition of embryogenic competence by cells, as earlier described (Puigderrajols et al. 2001; Verdeil et al. 2001). Ultrastructural changes in cytoplasm of cells connected with their individualisation consisted in increase of activity of Golgi structures. Formation of dictyosomal vesicles and their inclusion into cell walls is directly connected with changes in the cell walls, i.e., with disappearance of plasmodesms, thickening of the cell walls and disturbance in simplistic flow of nutritive substances (Verdeil et al. 2001). However, modifications in cell wall structure during isolation of cells at the stage of their dedifferentiation and isolation during entering somatic embryogenesis may vary. Both in direct somatic embryogenesis in roots of *Cichorium* (Dubois et al. 1990) and in indirect one in immature floral meristems of coconut (Verdeil et al. 2001), it has been shown that callose is deposited in thickening cell walls. Authors treat it as an early marker of somatic embryogenesis. Modifications of walls in the studied explant are accompanied by changes in shape and structure of nuclei and nucleoli. A symptom of increasing activity of metabolism in cells are enlarged, centrally located nuclei of granular structure, containing large nucleoli with "nucleolar vacuoles" inside. Such structure is an evidence of increased synthesis of RNA and is typical for cells getting ready for cell divisions (Johnson 1969) or for embryogenic callus tissue formation (Verdeil et al. 2001).

In the present work, the process of isolation was accompanied by dedifferentiation of cortex cells, followed by their individual divisions, leading in effect only to formation of callus tissue. Although structures observed on the fifth day of culture resembled proembryos by their shape and sequence of the first divisions, the process of somatic embryogenesis was not continued in those conditions and somatic embryos did not develop.

Initiation of callus results in changes of metabolism of nucleic acids and proteins, as well as lipids and carbohydrates. Storage materials occurring in tissues of the explant get hydrolysed during initiation of the divisions (Israel and Steward 1967), and other bodies are accumulated in newly-formed callus tissues. An example for that phenomenon is the gradual hydrolysis of lipid bodies in isolated zygotic embryo of *G. punctata* during its dedifferentiation on induction medium (Mikuła et al. 2000). Initial cells of all types of tissues of zygotic embryo were abundant in lipid bodies, whereas, about the 14th day of culture, lipid bodies in callus occurred occasionally, while amyloplasts were numerous. In studied root explants of *G. cruciata*, a gradual appearance of amyloplasts in cells was observed, preceding the initiation of cell divisions.

The development disturbed by exogenous plant growth regulator led to initiation of cell divisions in particular tissues of the studied explant, which later on formed callus tissue. Most of pericycle cells divided once of the third day of culture, while cortex managed to produce aggregates of 2-6 cells, giving the callus a specific appearance and character. It was already shown, that the manner and intensity of the reaction of particular tissues of the root explant dependent on, in majority, used plant growth regulators. The cells of pericycle of *Pisum sativum* root passed regular cell division on cytokinin-free medium, but supplemented with auxins. However, the used kinetin stimulated the cell divi-

sion of the cortical cells in 1 mm-thick segment root. Most of the cortical cells divided between 3rd and 5th day of culture and later formed callus tissue (Torrey and Fosket 1970). The structure of callus tissue formed by dividing cortex cells of *G. cruciata*, due to its separation may be called "aggregate". The formed type of callus made the tissue look nodular, crumbling when touched. The callus had loose, friable structure, was yellowish and developed quickly. Explants producing callus were, after several days of culture, often surrounded by a watery, sticky "matrix" consisting of single cells of the explant or callus (Mikuła 1998). A similar to the above described, "aggregate" type of divisions in the primary cortex, leading to formation of callus, was found in zygotic embryos of *G. punctata* (Mikuła et al. 2000). In that case, the callus tissue originated from epidermis, subepidermis and cortex cells. Cells of pericycle and axial cylinder were strongly vacuolated and decayed gradually. On the contrary, the discussed results show that the seedling root-derived callus of *G. cruciata* originated of cortex, pericycle and axial cylinder cells; decay affected epidermis and endoderm.

LITERATURE CITED

- DUBOIS T., GUEDIRA M., DUBOIS J., VASSEUR J. 1990. Direct somatic embryogenesis in roots of *Cichorium*: is callose an early marker? *Ann. Bot.* 65: 539-545.
- FILEK M., DUBERT F., MARCIŃSKA I., BIESAGA-KOŚCIELNIAK J. 1994. Zmiany potencjału elektrycznego podczas odróżnicowywania komórek i indukcji kalusa w eksplantach pszenicy. *Prace Ogródu Botanicznego PAN* 5/6: 333-337.
- FRANSZ P.F., SCHEL J.H.N. 1991. Cytodifferentiation during the development of friable embryogenic callus of maize (*Zea mays*). *Can. J. Bot.* 69: 26-33.
- HOSOKAWA K., NAKANO M., OIKAWA Y., YAMAMURA S. 1996. Adventitious shoot regeneration from leaf, stem and root explants of commercial cultivars of *Gentiana*. *Plant Cell Rep.* 15: 578-581.
- ISRAEL H.W., STEWARD F.C. 1967. The fine structure and development of plastids in cultured cells of *Daucus carota*. *Ann. Bot.* 31: 1-18.
- JOHNSON J.M. 1969. A study of nuclear vacuoles in cultured tobacco cells using radioautography Actinomycin D, and electron microscopy. *J. Cell Biol.* 43: 197-206.
- KONAR R.N., THOMAS E., STREET H.E. 1972. Origin and structure of embryoids arising from epidermal cell of the stem of *Ranunculus sceleratus* L. *J. Cell Science* II: 77-93.
- MASUDA Y. 1977. Hormone interactions in regulation of cell wall loosening. In: Schütte H.R. Gross D. (Eds): Regulation of developmental processes in plants. *Proceedings Conference Halle July 4th-9th 1977*. Pp319-330.
- MIKUŁA A. 1998. Embriogeniczny charakter kultur *in vitro* wybranych gatunków z rodzaju *Gentiana*. *Praca doktorska. Ogród Botaniczny CZRB PAN*.
- MIKUŁA A., RYBCZYŃSKI J. 1999. Present status of *Gentiana* taxa biotechnology – A review. *Biotechnologia* 4: 1-16.
- MIKUŁA A., RYBCZYŃSKI J. 2001. Somatic embryogenesis of 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). *Acta Physiol. Plant.* 23: 15-25.
- MIKUŁA A., RYBCZYŃSKI J., TYKARSKA T., KURAŚ M. 2001. Somatic embryogenesis of *Gentiana* genus II. Scanning and ultrastructural analysis of early stages of somatic embryogenesis in liquid medium. *Biol. Bull. Poznań* 38: 47-53.

- MIKUŁA A., TYKARSKA T., ZIELIŃSKA M., RYBCZYŃSKI J., KURAŚ M. 2000. Ultrastructure of initial stages of tissue proliferation of *Gentiana punctata* zygotic embryo on induction medium. IX. Ogólnopolska Konferencja Kultur in vitro i Biotechnologii Roślin „Modyfikowanie Genomu Roślin”, Gdańsk–Sobieszewo 10-13 IX 2000, s. 115.
- MIKUŁA A., WESOŁOWSKA M., KAPUSTA J., SKRZYP-CZAK L., RYBCZYŃSKI J. 1996. Cytomorphological studies on somatic embryogenesis of *Gentiana tibetica* (King) and *G. cruciata* (L.). Acta Soc. Bot. Pol. 65: 47-51.
- MOMČILOVIĆ I., GRUBIŠIĆ D., NEŠKOVIĆ M. 1997. Micro-propagation of four *Gentiana* species (*G. lutea*, *G. cruciata*, *G. purpurea* and *G. acaulis*). Plant Cell Tiss. Org. Cult. 49: 141-144.
- MURASHIGE T., SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
- NISHITANI K., SHIBAOKA H., MASUDA Y. 1979. Growth and cell wall changes in azuki bean epicotyls. II. Changes in wall polysaccharides during auxin-induced growth of excised segments. Plant Cell Physiol. 20: 463-472.
- PUIGDERRAJOLS P., MIR G., MOLINAS M. 2001. Ultrastructure of early secondary embryogenesis by multicellular and unicellular pathways in Cork Oak (*Quercus suber* L.) Ann. Bot. 87: 179-189.
- REYNOLDS E.S. 1963. Use of lead citrate at high pH as electron opaque stain in electron microscopy. J. Cell Biol. 17: 208-213.
- STREET H.E. 1978. Differentiation in cell and tissue cultures – regulation at the molecular level. [in:] H.R. Schutte, D. Gross (red.) Regulation of developmental processes in plants. Proceed. Conf. Halle, 4-9 July 1977; 192-218.
- TORREY J.G., FOSKET D.E. 1970. Cell division in relation to cytodifferentiation in cultured pea root segment. Am. J. Bot. 57 (9): 1072-1080.
- CONGER B.V., MCDANIEL J.K. 1989. Origin of direct somatic embryos from cultured leaf segments of *Dactylis glomerata*. Bot. Gaz. 150 (1): 72-77.
- VERDEIL J.L., HOCHER V., HUET C., GROSDEMANGE F., ESCOUTE J., FERRIERE N., NICOLE M. 2001. Ultrastructural changes in coconut calli associated with the acquisition of embryogenic competence. Ann. Bot. 88: 9-18.
- WILLIAMS E.G., MAHESWARAN G. 1986. Somatic embryogenesis: factors influencing coordinated behaviour of cell as an embryogenic group. Ann. Bot. 57: 443-462.

ANALIZA ULTRASTRUKTURALNA POCZĄTKOWYCH STADIÓW ODRÓŻNICOWANIA EKSPLANTATÓW KORZENIOWYCH SIEWEK *GENTIANA CRUCIATA*

STRESZCZENIE

Badania prowadzono na izolowanych korzeniach 10-dniowych siewek *Gentiana cruciata*, które hodowano na pożywce Murashige i Skoog (1962) zawierającej 1,0 mg/dm³ Dicamba + 0,1 mg/dm³ NAA + 2,0 mg/dm³ BAP + 80,0 mg/dm³ siarczanu adeniny. Stosując mikroskop świetlny i elektronowy obserwowano zmiany jakie zachodziły w eksplantatach między 3 a 11 dniem kultury. Obserwowano stopniowe odróżnicowywanie się tkanek korzenia, które najwcześniej uwidoczniło się w korze na granicy cięcia eksplantatu i kolejno przemieszczało się w głąb korzenia, obejmując jednocześnie coraz niższe partie jego strefy elongacyjnej. Najintensywniejsze tworzenie tkanki kalusowej zachodziło w miejscu cięcia korzenia, w którym proliferacja komórek objęła zarówno korę jak i walec osiowy. W apikalnej części strefy elongacyjnej podziały zachodziły jedynie w endodermie i w walcu osiowym, podczas gdy część merystematyczna korzenia była nieaktywna. Ostatecznie w badanym eksplantacie wyróżniono następujące strefy: (I) intensywnych podziałów komórkowych obejmującą część elongacyjną korzenia, (II) strefę dyspersji komórek oraz (III) nieaktywnego merystemu.

Przejawem gotowości komórek do podjęcia podziałów było przeorganizowanie ich ultrastruktury. Obserwowano wzmożoną aktywność mitochondriów i struktur Golgiego, pogrubianie się ścian i zanikanie plasmodesmowych połączeń oraz pojawianie się amyloplastów i ciał lipidowych w tkankach, w których dotychczas one nie występowały, bądź występowały sporadycznie. Intensywnie dzielące się komórki nabywały cech komórek merystematycznych. Wykazywały one gęstą cytoplazmę z licznymi organellami, duże centralnie położone jądro, które zawierało wakuole jądrowe wewnątrz jąder. Tkanka kalusowa powstała z komórek kory miała agregatowy charakter, natomiast z komórek perycyklu i endodermy powstawał kalus o charakterystycznym zwartym układzie i regularnym typie podziałów.

SŁOWA KLUCZOWE: korzenie siewek *Gentiana cruciata*, odróżnicowanie, ultrastruktura, rozwój kalusa.