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ULTRASTRUCTURAL CHANGES IN ZYGOTIC EMBRYOS OF *GENTIANA PUNCTATA* L. DURING CALLUS FORMATION AND SOMATIC EMBRYOGENESIS

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Imbibed immature zygotic embryos of *Gentiana punctata* L. were cultured on MS (Murashige and Skoog) medium consisting of 4.5 μM dicamba, 0.54 μM NAA (naphtaleneacetic acid), 8.88 μM BAP (6-benzylaminopurine) and 0.43 mM AS (adenine hemisulfate). The primary response of explants consisted in thickening of the subcotyledon and hypocotyl root (HR) zone. Cotyledons and the seminal root did not show any response. Ultrastructural analysis of the initial stages of callus formation revealed numerous changes in cells of explants. Dedifferentiation of the explant tissues was associated with separation of cells resulting from thickening and folding of walls, destruction of plasmodesmata, and enlargement of intercellular spaces. At the same time, the number of lipid bodies decreased and starch appeared. Indicative of changes in 3-day cultures, the first cell divisions were observed to occur in the HR zone, including cells of the primary cortex, endodermis and pericycle. The dividing cells contained small vacuoles, large, centrally located, layered nuclei with vacuolated nucleoli, amyloplasts with starch, lipid bodies, numerous active Golgi structures, mitochondria and rough endoplasmic reticulum. Actively dividing cells formed callus tissue in which three zones of cells could be distinguished after 14 days of culture: (I) outer (starch) layer, (II) middle layer with actively at dividing small cells, and (III) inner layer containing large vacuolated cells. As the result of cell divisions, at about the fourth week of culture the starch zone formed meristematically active centers of small cells, with dense cytoplasm and large amounts of starch. Among them were small cellular complexes consisting of three cells, with the cell wall structure typical for pre-embryos. By the fifth week of culture, numerous globular and early heart-shaped somatic embryos which formed cotyledons were observed, and further mature somatic embryos showing conversion ability.

Key words: *Gentiana punctata* L., zygotic embryo, ultrastructure, dedifferentiation, callus development, somatic embryo.

INTRODUCTION

So far, clonal plants of the genus *Gentiana* have been regenerated in culture in vitro mainly by organogenesis (Skrzypczak et al., 1993; Mikuła and Rybczyński, 1999; Momčilowić et al., 2001). Knowledge of somatic embryogenesis in these species is still insufficient. However, our studies of somatic embryogenesis of *G. cruciata* L., *G. pannonica* Scop. and *G. tibetica* King showed that this method of plant propagation can be used with gentian to facilitate its long-term and effective regeneration (Mikuła and Rybczyński, 2001; Mikuła et al., 2001, Mikuła et al., 2002a). In *Gentiana* the degree of differentiation of the explant seems to be important to the course of plant regeneration. So far, gentian

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plants have been regenerated by organogenesis from explants of leaves (Hosokawa et al., 1996), shoots (Sharma et al., 1993; Hosokawa et al., 1996; Momčilowić et al., 1997), roots (Hosokawa et al., 1996), buds (Yamada et al., 1991), axillary shoots (Morgan et al., 1997) and apical meristems (Sharma et al., 1993). The use of younger explants, that is, fragments of seedlings of *G. cruciata*, *G. pannonica* and *G. tibetica* (Mikuła and Rybczyński, 2001) and apical meristems of *G. pneumonanthe* (Bach and Pawłowska, 1994) allowed induction of somatic embryogenesis in those species. Some of the explants, for example those originated from root, were not able to regenerate embryogenic but only meristematic tissue; both central cylinder and cortex tissues responded.

Gentiana punctata is another species which has shown morphogenic potential in tissue culture systems. Cotyledons, hypocotyls, roots (Skrzypczak et al., 1993) and epicotyls of *G. punctata* seedlings (Vinterhalter and Vinterhalter, 1998) allowed regeneration of plants by organogenesis alone. Explants of zygotic embryos have not yet been used before in gentian biotechnology. The zygotic embryo, as an explant ontogenetically younger than any seedling organ (Williams and Maheswaran, 1985) of *G. punctata* could serve as a potential source of embryogenic tissue.

Somatic embryogenesis is a biological phenomenon much exploited for propagation of various plant species. In this process it is important to study the initial stages of the changes that ultimately produce a character of biological value, which can be compared to the potential of the zygote. These changes can be studied on the ultrastructural level. Here we use light and electron microscopy to examine changes in tissues and cells of the mature zygotic embryo leading to the formation of embryogenic callus and somatic embryos.

MATERIALS AND METHODS

PLANT MATERIAL

Mature zygotic embryos of *Gentiana punctata* L. about 1 mm long were isolated from seeds (Fig. 1) collected in Tatra National Park, Poland, and used as explants.

CULTURE CONDITIONS

The seeds were immersed in 70% ethanol for a few seconds and disinfected with a 10% (v/v) solution of Domestos bleach (active product: sodium hypochlorite) for 20 min, then rinsed three times in sterile distilled water. The disinfected seeds were stratified for 14 days at 5˚C. Then, after 2 h of imbibition in sterile water, whole embryos were isolated and placed on initial MS medium (Murashige and Skoog, 1962) supplemented with 4.5 μM dicamba, 0.54 μM NAA, 8.88 μM BAP and 0.43 mM AS (adenine hemisulphate). The cultures were kept in a culture room at 22˚C with a 16 h photoperiod under white light (40 W fluorescent tube; 100 $\mu Em^{-2}s^{-1}$).

SAMPLING OF PLANT MATERIAL

For microscopic analyses, explants were collected after 2 h of imbibition (control), and then every 2 days for 14 days and after 3, 4 and 5 weeks of culture. Whole embryo axes were fixed for observations by light and transmission electron microscopy. All specimens presented in this work show the same part of the embryo: the border zone between the hypocotyl and root (HR zone). For all explants collected at different intervals of culture, both longitudinal and cross sections were made.

Macroscopic observations were continued until the cotyledon stage of somatic embryos and their subsequent conversion into young plantlets.

MICROSCOPIC PREPARATIONS

Samples of material were fixed in 2% glutaraldehyde (pH 7.2) in 0.1 M cacodylate buffer (pH 7.2) for 2 h followed by additional fixation in 2% OsO_4 for 2 h at 4˚C. They were then dehydrated in a graded series of ethanol and propylene oxide and embedded in a mixture of Epon and Spurr. Semithin (1–2 μm) and ultrathin (0.6 μm) sections were cut with an ultramicrotome (LKB, Sweden). Semithin sections were stained with 0.1% toluidine blue in 1% borax and analyzed with a Navox-Olympus light microscope. Ultrathin sections were contrasted in solutions of uranyl acetate (30 min) and lead citrate (30 min) according to Reynolds (1963). Specimens were observed with a JEOL JEM 100C transmission electron microscope.

RESULTS

MACROSCOPIC ANALYSIS OF EXPLANTS

Upon culture, isolated zygotic embryos of *G. punctata* (Fig. 1) showed the most intensive changes in their subcotyledon and HR zone (Fig. 2). Both parts

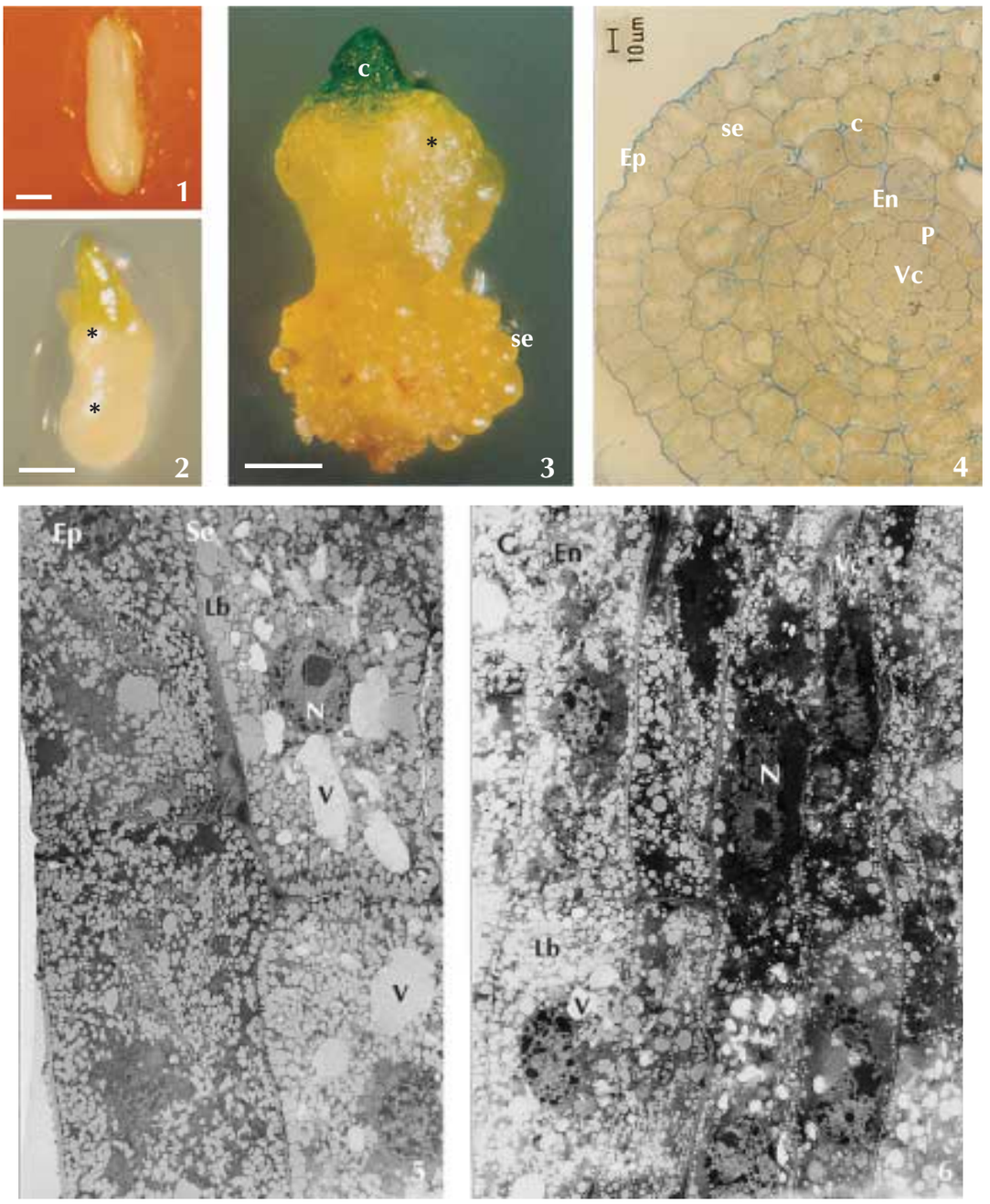


Fig. 1. Mature zygotic embryo of *G. punctata* isolated after 2 h of seed imbibition (in vivo). Bar = 0.2 mm. **Fig. 2.** Zygotic embryo after 2 weeks of culture on the medium. Note thickenings of the subcotyledon and hypocotyl root part (asterisks). Bar = 0.5 mm. **Fig. 3.** Embryo after 5 weeks of culture on the medium. Note intensively green cotyledons (C), thickened subcotyledon part (asterisk) and callus tissue; somatic embryos (se) are seen in the border zone between hypocotyl and root. Bar = 1.0 mm. **Fig. 4.** Cross section through the HR zone of the zygotic embryo after 2 h of imbibition. Ep – epidermis; Se – subepidermis; C – cortex; En – endodermis; P – pericycle; Vc – axial cylinder (semithin section). **Fig. 5.** Ultrastructure of epidermal (Ep) and subepidermal (Se) cells of control zygotic embryo after 2 h of imbibition. Cells are abundantly filled with lipid bodies (Lb) and vacuoles (V) of different sizes. × 1300. **Fig. 6.** Ultrastructural view of cortex (C), endodermis (En), pericycle (P) and axial cylinder (Vc) cells of control embryo. Note numerous lipid bodies (Lb), nuclei (N) and small vacuoles (V) (longitudinal section). × 1300.

were enlarged; however, callus did not appear in the subcotyledon zone. Its formation was limited to the intensively thickening HR zone in which somatic embryos were also seen at about five weeks of culture (Fig. 3). The embryo cotyledons turned green but did not show any other response (Figs. 2, 3), and the meristematic part of the root underwent necrosis (Fig. 3).

MICROSCOPIC ANALYSIS OF EXPLANTS

Analysis of control explants

Microscopic analysis of the HR zone of a zygotic embryo isolated after 2 h of imbibition showed typical histological differentiation into the epidermis, primary cortex and axial cylinder (Fig. 4). Epidermal cells were radially elongated and closely packed; cortex cells were large and cylindrical, with small intercellular spaces. Endodermal cells were smaller, closely packed, and flattened parallel to the surface. Cells of the axial cylinder were small, polyhedral, closely packed, and surrounded by a separate layer of pericycle cells (Fig. 4).

The cells of all tissues contained dense cytoplasm and a centrally located nucleus, and were filled with large amounts of lipid bodies; starch grains were missing (Figs. 5, 6). Cells of the cortex and axial cylinder contained a few small vacuoles; they were slightly larger only in the subepidermis (Fig. 5).

Analysis of explants during subsequent days of culture

During culture, the following stages of responses in the HR zone of the investigated explant were observed: (1) dedifferentiation of explant tissues (1–3 days of culture), (2) formation of callus tissue (from the third day of culture), (3) differentiation of the starch zone (from the fourth day of culture), and (4) differentiation of somatic embryos (from the fourth week of culture).

Dedifferentiation of the cells of cultured embryos (stage 1) was initiated by thickening and folding of cell walls and enlargement of intercellular spaces. All tissues of the HR zone underwent this process (Figs. 7, 8); at the same time, epidermal cells contacting the medium died (Fig. 9). On the second day of culture, the number of lipid bodies was reduced. Starch grains appeared in the cells, and vacuoles contained deposits of electron-dense bodies (Fig. 7). The cytoplasm contained numerous mitochondria of different shapes, and rough endoplasmic reticulum (Fig. 10). The cell walls, which were straight in control cells (Figs. 5, 6), became folded (Figs. 7, 8) but they still contained numerous plasmodesmata (Fig. 7). The observed changes eventually led to their destruction and to isolation of the explant cells. After those events, cell divisions appeared (Fig. 11), marking the beginning of callus formation. The first divided cells were observed as early as the third day of culture in the cortex (Fig. 11), epidermis and pericycle (Fig. 12).

After four days of culture (stage 2), many divided cells were observed in the cortex (Figs. 13, 14), endodermis and pericycle (Figs. 13, 15). They contained numerous Golgi structures and lipid bodies; large amounts of starch were found in the callus formed in the cortex (Fig. 14). The endodermis, consisting of one layer in the control (Fig. 4), now had several layers as a result of periclinal and anticlinal divisions (Figs. 13, 15). Cells directly contacting the medium (epidermis) and located nearby (cortex, to the axial cylinder) underwent gradual degradation (Fig. 16). Cortical cells adjacent to necrotic cells were large and vacuolated, while cells lying away from the medium were small and dividing intensively. They contained numerous amyloplasts with starch (Figs. 16, 17), lipid bodies and large layered nuclei, the nucleoli of which had nucleolar vacuoles (Fig. 18). The walls of newly divided cells contained plasmodesmata (Fig. 19), whereas older walls were markedly thickened, multi-layered, folded and without plasmodesmata (Fig. 20). The cytoplasm was rich in dictyosomes, mitochondria and rough endoplasmic reticulum (Fig. 21). Active Golgi structures produced numerous dictyosomal vesicles. Actively dividing cortical cells formed callus tissue.

Starting from the eighth day of culture, cells within the callus tissue differentiated (stage 3). Later on, three zones of cells could be distinguished within the callus: I. Outer (starch) zone, consisting of cells rich in starch (Figs. 22–24); II. Middle zone, with small, actively dividing cells (Figs. 22, 25); III. Inner zone, containing large vacuolated cells (Figs. 22, 26).

The starch zone had 2–4 layers of uniform cells with numerous large amyloplasts (Figs. 23, 24). Small cells of the middle zone (Figs. 24, 25) contained large centrally located nuclei, often having vacuolated nucleoli, dense cytoplasm, small starch grains and very numerous but small vacuoles. Cells inside the explant, that is, cells of the axial cylinder and cortex cells in its neighborhood, underwent vacuolization, forming the inner zone (Fig. 26). Large vacuoles contained numerous deposits of electron-

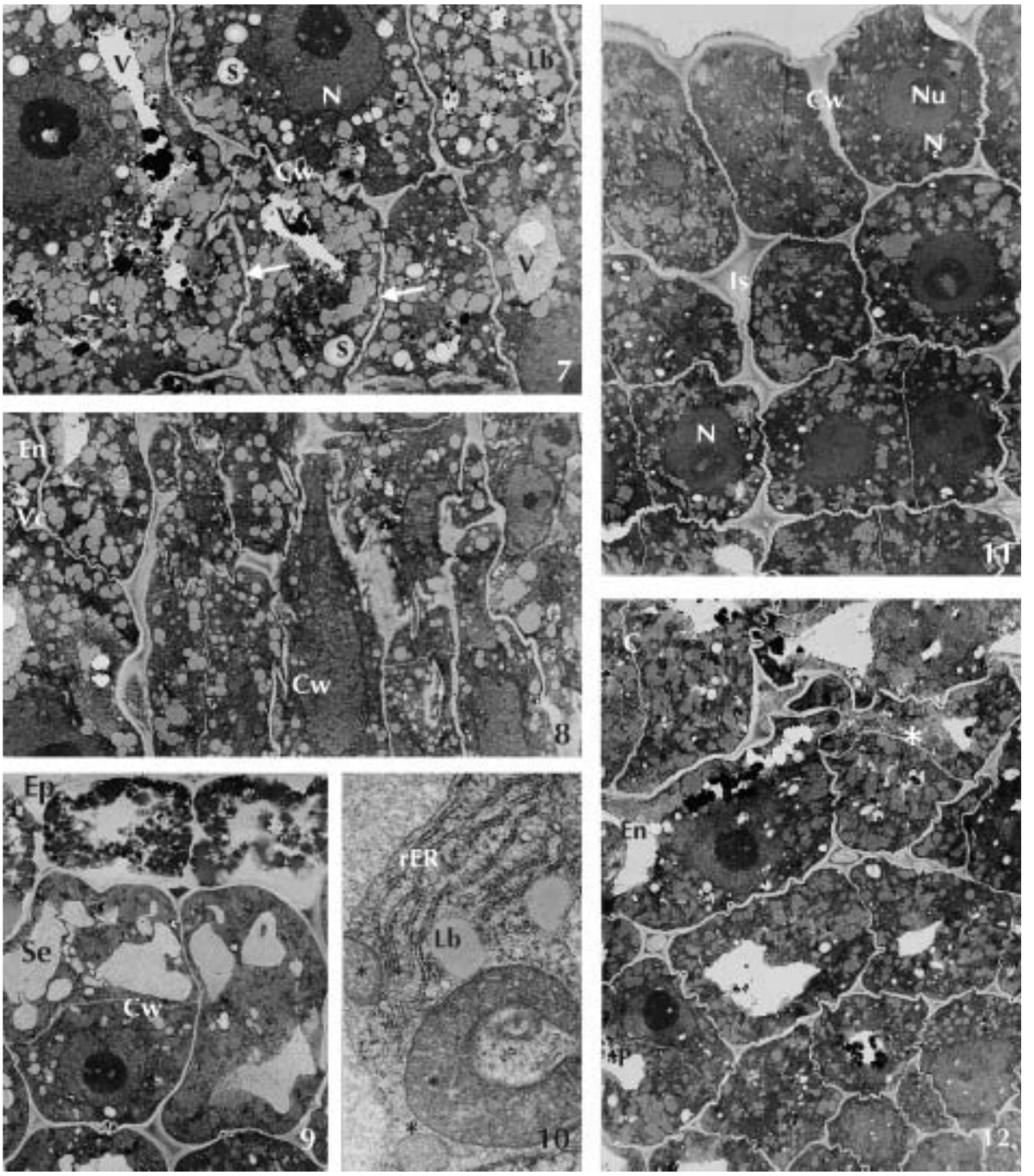


Fig. 7. Changes in primary cortical cells after 2 days of culture: folded cell walls (Cw) with plasmodesmata (arrows), numerous lipid bodies (Lb), starch grains (S) and vacuoles (V) (longitudinal section). × 1800. **Fig. 8.** Cells of endodermis (En) and axial cylinder (Vc) after 2 days of culture: folded cell walls (Cw). × 1500. **Fig. 9.** Necrosis of epidermis cells (Ep) in contact with medium, division in subepidermis (Se). Cw – young cell wall (3rd day of culture) (cross section). × 1400. **Fig. 10.** Numerous rough endoplasmic reticula (rER), mitochondria (asterisks) and lipid bodies (Lb) in dividing cortical cells. × 14000. **Fig. 11.** Thick folded cell wall (Cw), isolation of particular cells of explant before their division (3rd day of culture). Is – intercellular spaces; Lb – lipid bodies; N – nucleus; Nu – nucleolus (cross section). × 1200. **Fig. 12.** Initial divisions of endodermis (En) and pericycle (P) cells (3rd day of culture). C – primary cortex; asterisk – divided cell (cross section). × 1200.

dense substance. The degree of vacuolization of cells inside the explant gradually increased.

As a result of subsequent divisions, outer cells of the starch-containing zone formed a meristematic zone inside the callus tissue, where differentiation of somatic embryos took place (Fig. 26). About the fourth week of culture (stage 4), in zone I (starch zone) of the callus tissue, meristematically active centers of small cells were observed (Fig. 27). The cells contained dense cytoplasm and numerous starch grains. The concentrations of those cells gave the callus an aggregate structure. The aggregates contained cells which underwent two initial divisions typical for proembryo formation. The first one was a cross division resulting in the formation of apical and basal cells. The second cell division was perpendicular to it, taking place in the apical cell.

The proembryos, formed by further cell divisions, were separated from adjacent cells by a thick wall. Their cells were closely packed, with dense cytoplasm, and a suspensor was distinguished (Figs. 27–29). Cells at the base of the embryo were large, polyhedral, strongly vacuolated and less stainable (Fig. 29). In the fifth week of culture there were numerous globular and heart-shaped somatic embryos on the callus surface (Fig. 29). Subsequently they developed into heart-shaped embryos with distinctly formed leaf primordia. A hollow in the apical meristem was noted (Fig. 30). Formation of cotyledon-stage embryos and their conversion into young plants completed the evidence of somatic embryogenesis in *G. punctata*.

DISCUSSION

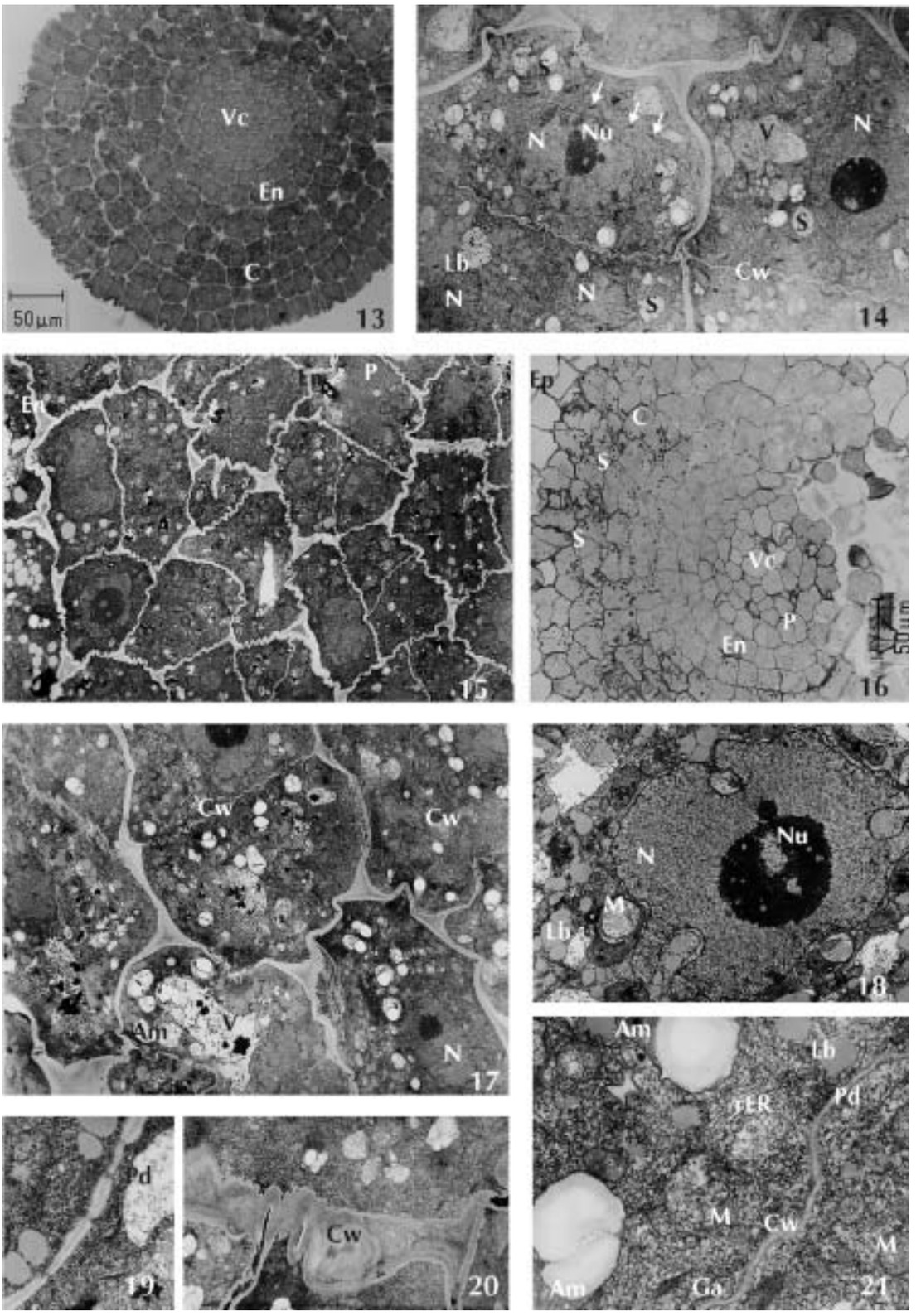
On the basis of several years of experience in tissue culture of gentian with the use of various type of explants, we chose the mature zygotic embryo of *G. punctata* for culture initiation. We developed culture conditions enabling induction of somatic embryos, and focused mainly on changes in ultrastructure.

Induction of embryogenesis in vitro is highly dependent on the type and age of the explant (Maheswaran and Williams, 1985; Tenning et al., 1992). The subcotyledon and HR zone of the *G. punctata* embryo reacted to the applied conditions of culture. Dedifferentiation of tissues in the responsive areas took different courses. Parenchymal tissues of the subcotyledon part of the embryo were strongly vacuolated, forming thickened areas only, while callus tissue was formed at the HR zone.

The first response of tissues in the HR zone was isolation of particular cells of the primary cortex and epidermis, by thickening of walls, disappearance of plasmodesmata, and enlargement of intercellular spaces. Biochemical changes in cell walls (Nishitani et al., 1979) and their loosening, probably under the influence of hormones, allowed the growth of cells (Masuda, 1977). Loosening of the middle lamella resulted most likely from intensive synthesis and secretion of hydrolytic enzymes (Fransz and Schel, 1991). Those changes were accompanied by the disappearance of plasmodesmata, which caused a change from symplastic to apoplastic transport of nutrients (Fransz and Schel, 1991; Verdeil et al., 2001). Processes observed at the first stage of cell/tissue dedifferentiation somewhat resemble changes occurring during morphogenesis. They are a necessary condition for totipotency and one of the initial responses of cells entering the somatic embryogenesis pathway. Isolation of competent embryogenic cells is accompanied additionally by modifications of cell wall composition, such as the appearance of callose (Dubois et al., 1990; 1991; Verdeil et al., 2001) or cutin (Pedroso and Pais, 1995).

Physiological isolation of cells is necessary for the emergence of somatic embryos from explant cells (Maheswaran and Williams, 1985; Dubois et al.,

Fig. 13. Numerous divided cells of cortex (C) and endodermis (En). Vc – axial cylinder (4th day of culture) (cross section; semithin section). **Fig. 14.** Divided primary cortical cell with typical folding of cell wall (Cw), numerous lipid bodies (Lb), starch (S) and Golgi structures (arrows) (4th day of culture). N – nucleus; Nu – nucleolus. × 2000. **Fig. 15.** Multilayered endodermis (En) and divided pericycle (P) cells (4th day of culture; cross section). × 1200. **Fig. 16.** Changes in explant after 6 days of culture. Numerous starch grains (S) in divided cortex (C) cells, except in cells of endodermis (En). Note necrosis of tissues in contact with medium and epidermis on its whole circumference. P – pericycle, Vc – axial cylinder (semithin section). **Fig. 17.** Ultrastructure of cortical cells after 6 days of culture: large, centrally located nuclei (N), numerous starch grains in amyloplasts (Am), vacuoles with electron-dense deposits (V), thin walls of newly divided cells and thick walls between neighboring cells (Cw). × 1500. **Fig. 18.** Large layered nucleus (N) with nucleolus (Nu) containing vacuoles and numerous lipid bodies (Lb) and mitochondria (M) in cortex cell (6th day of culture). × 4100. **Fig. 19.** Numerous plasmodesmata (Pd) between neighboring newly divided cells (6th day of culture). × 8200. **Fig. 20.** Strongly thickened, folded, layered cell wall (Cw) (6th day of culture). × 6000. **Fig. 21**. Rich cytoplasm of cortical cells: active Golgi structures (GA), mitochondria (M), rough endoplasmic reticulum (rER), amyloplasts (Am) and plasmodesmata (Pd) in cell wall (Cw) (6th day of culture). × 6500.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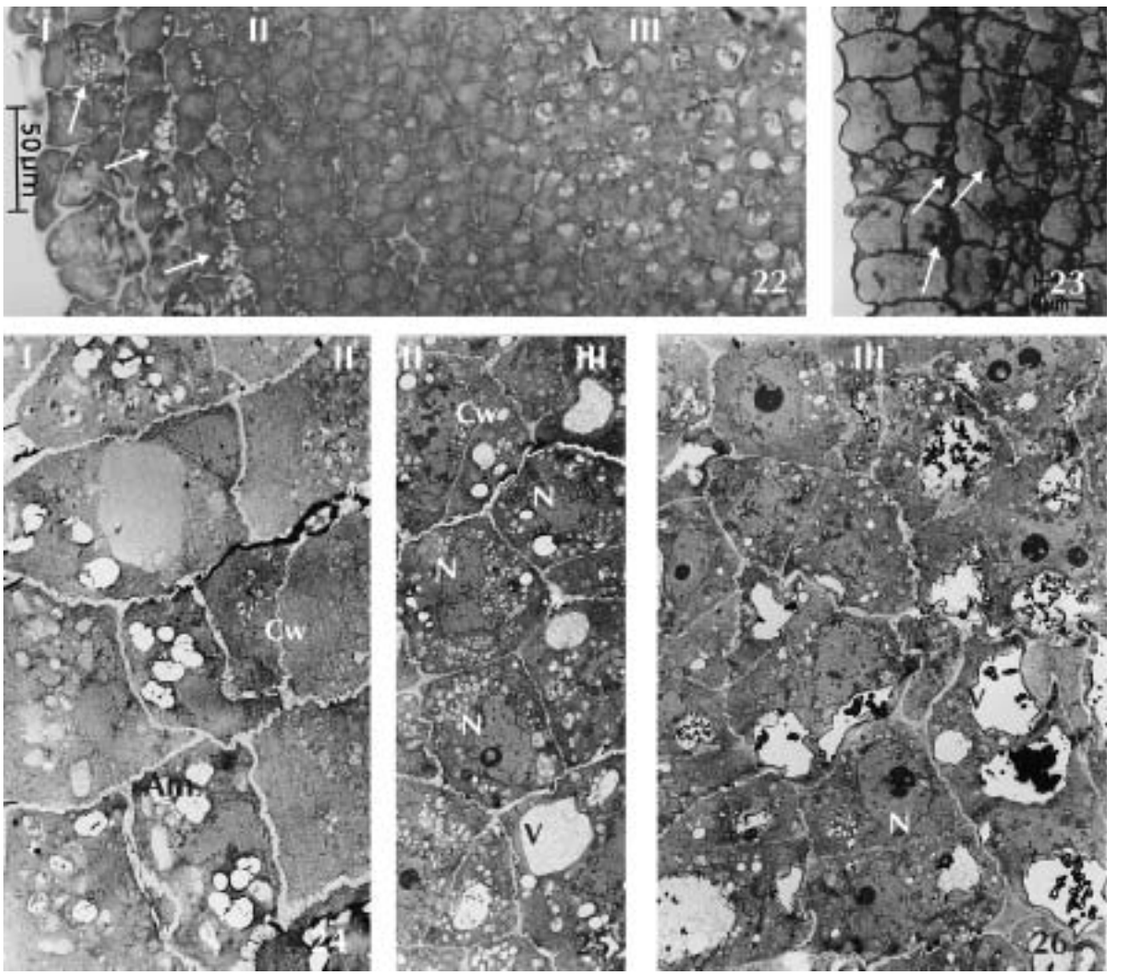
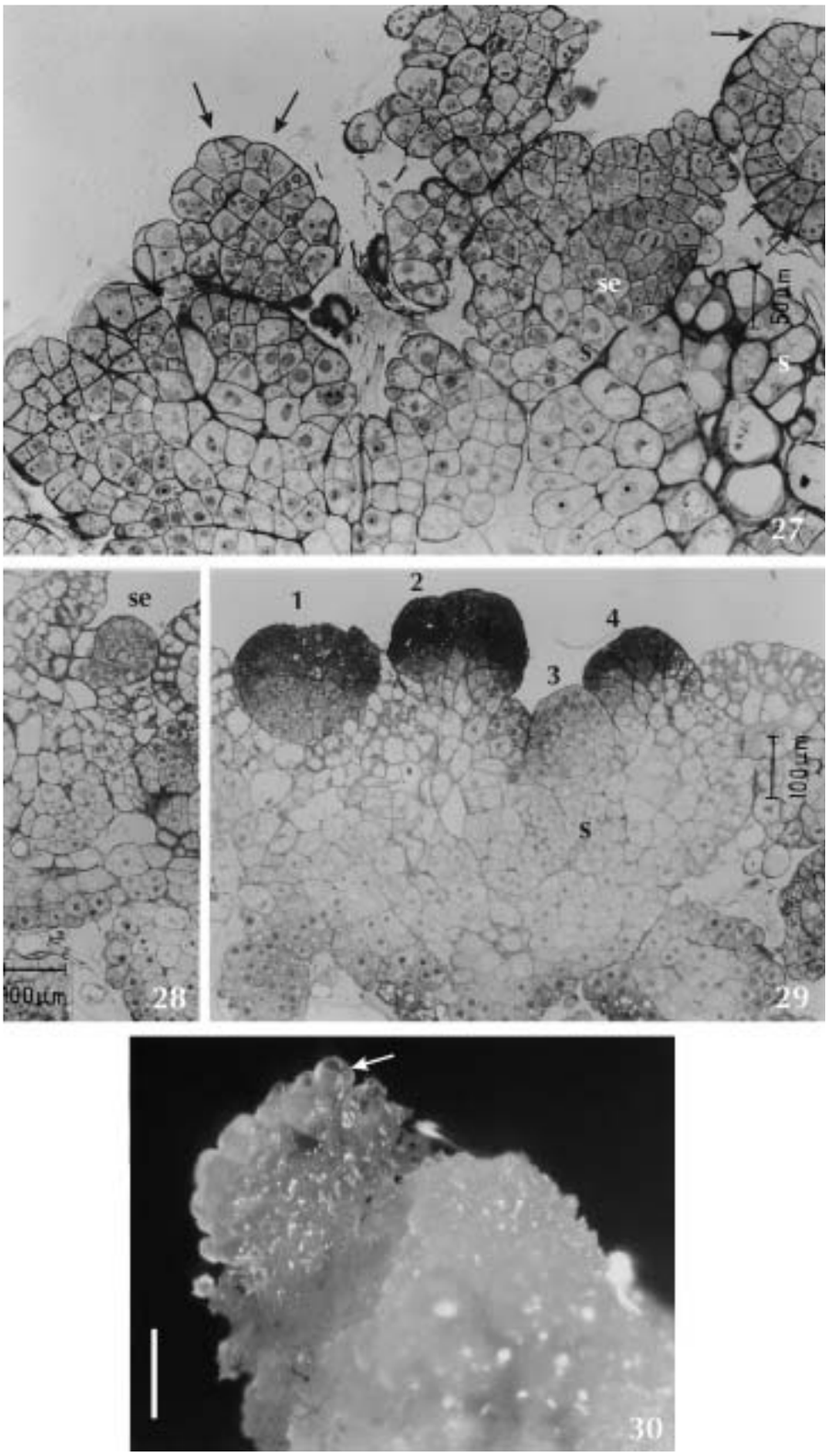


Figs. 22–26. Longitudinal section through 14-day-old explant in hypocotyl root part. **Fig. 22.** Formation of three zones in callus: I – starch zone (arrows – amyloplasts); II – intensively dividing small cells; III – cells undergoing vacuolation (semithin section). **Fig. 23.** Peripheral cells of callus tissue, rich in starch (arrows) – zone I (semithin longitudinal section). **Fig. 24.** Ultrastructure of cells of zone I at the border of zone II, containing numerous amyloplasts (Am) with starch. × 1000. **Fig. 25.** Ultrastructure of actively dividing cells of zone II at the border of zone III – large, centrally located nuclei (N) with vacuolated nucleoli, thin cell walls (Cw). V – vacuole. × 1000. **Fig. 26.** Ultrastructure of vacuolated cells of zone III. V – vacuole; N – nucleus. × 1000.

1991; Canhoto et al., 1996), from callus tissue (Puigderrajols et al., 2001; Mikuła et al., 1996a; Mikuła et al., 1996b; Verdeil et al., 2001), and during secondary somatic embryogenesis (Canhoto et al., 1999). Yeung (1995) also suggests that the physical and physiological isolation that results from thickened cell walls and from the lack of plasmodesmata, which leads to the absence of symplastic transport, plays an important role in the formation of somatic embryos. Isolation of cells in primary explants also seems to be a condition for induction of a change in their previous direction of development. Isolation of seedling root explant cells at the stage of their dedifferentiation was also observed in *G. cruciata* (Mikuła et al., 2002). In this case the process of physiological isolation took place in cortex cells, which further underwent individual divisions; on the fifth day of culture they formed structures resembling proembryos in shape and in the sequence of initial divisions. However, these structures did not develop into somatic embryos; they produced nonembryogenic callus tissue. Another manner of dedifferentiation was observed in cells of the endodermis and pericycle of *G. cruciata* root. Dividing cells formed a multilayered,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dense-structured zone of meristematic type, without previous isolation of cells as is typical for cortex.

As seen in the seedling root explant of *G. cruciata* (Mikuła et al., 2002b), isolation of the cortex cells of a zygotic embryo of *G. punctata* was accompanied by divisions leading to the formation of callus tissue. Ultrastructural observations revealed increasing metabolic activity and intensive RNA synthesis in cells (Johnson, 1969). These changes are typical for cells preparing to divide (Verdeil et al., 2001). Initiation of callus causes changes in the metabolism of nucleic acids, proteins, lipids and carbohydrates. Storage materials previously accumulated undergo hydrolysis during induction of divisions (Israel and Steward, 1967), and the newly formed callus tissue gathers different types of such materials. In our work we observed a gradual reduction of the number of lipid bodies and the deposition of starch in amyloplasts.

Gradual differentiation of several zones was observed within newly formed callus tissue of the *G. punctata* zygotic embryo. This is a typical phenomenon described by other authors and called the meristematic layer, meristematic zone, or proembryogenic cells (Pierson et al., 1983; Michaux-Ferrière and Carron, 1989; Canhoto et al., 1999; Verdeil et al., 2001). In this zone, somatic embryos are differentiated, and starch stored therein is utilized as a source of energy for intensively dividing cells and developing somatic embryos (Stamp, 1987; Barciela and Vieitez, 1993). Besides the starch zone, Pierson et al. (1983) identified a zone of small symmetric cells filled with dense cytoplasm, and also small vacuoles and scarce starch in the ultrastructure of *Gentiana* cells.

As in many other species (Pierson et al., 1983; Stamp, 1987; Cruz et al., 1990; Canhoto et al., 1996; Puigderrajols et al., 2001; Verdeil et al., 2001), in *G. punctata* the differentiation of somatic embryos occurred in the surface meristematic layer of cells. In our study, *G. punctata* did not require subculture or a change of medium to an auxin-free one. In conditions of an unchanged initial medium, globular embryos were obtained within five weeks of culture, undergoing subsequent developmental stages until the cotyledon stage. New embryos appeared constantly during the development and maturation of older ones.

Our results confirm those of other authors (Pierson et al., 1983; Stamp, 1987; Michaux-Ferrire and Carron, 1989; Canhoto et al., 1999; Puigderrajols et al., 2001; Verdeil et al., 2001) on zonal differentiation of cells within the callus tissue. The layer of embryogenic cells formed in its peripheral part was the site of constant formation of somatic embryos. The phenomenon of isolation of cells, described earlier as necessary for the emergence of embryogenic totipotency, occurs in *G. punctata* also. However, it seems that isolation of cells is an inevitable condition as early as the stage of their dedifferentiation in the explant.

This study provided evidence that somatic embryogenesis in *G. punctata* is related to the response of primary tissues of a particular part of the mature embryo. The cells of the cultured embryo divided, resulting in the formation of embryogenic callus. Indirect induction of somatic embryos in the starch layer of callus confirmed earlier-described results on tissue culture of different explants of *Gentiana* species. Understanding the background of somatic embryogenesis will be useful for genome manipulation in this group of plants.

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Fig. 27. Differentiation of pre-embryos within meristematically active centers of cells (4th week of culture). Arrows – divisions initiating formation of somatic embryo: the first is longitudinal, the second is transverse. se – somatic embryo; s – suspensor (semithin section). **Fig. 28.** Semithin section of embryogenic tissue and somatic embryo (se) at globular stage. **Fig. 29.** Somatic embryos at globular (3,4) and early heart-shaped stage (1,2). s – suspensor (semithin section). **Fig. 30.** Numerous somatic embryos at globular and heart-shaped stages (arrow). Bar = 0.5 mm.

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