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Comparative Ultrastructural Analysis of the in vitro Microspore Embryoids and in vivo Zygotic Embryos of Wheat as a Basis for Understanding of Cytophysiological Aspects of Their Development

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Abstract—Ultrastructures of in vitro microspore embryoids and in vivo zygotic embryos of spring wheat have been analyzed and compared. Along with the similarity of ultrastructural characteristics of embryoid and embryo cells at the corresponding developmental stages, some differences have been revealed. Unlike embryos, embryoid cells are characterized by lipid inclusions and numerous mitochondria with well-developed internal membranes. According to our hypothesis, lipids represent an alternative energy source required for active cell divisions in the forming embryoids. Unlike embryos, since the earliest developmental stages, embryoid cells accumulate a significant amount of starch and then utilize it during the organogenesis and germination. A conclusion has been made that embryoid cells create their own reserve of carbohydrates, which is then mobilized during their development. The concept of T.B. Batygina (1987, 1997, 2014) about the universal character of the plant morphogenesis in vivo, in situ, and in vitro has been confirmed. The prospects for the use of microspore embryoidogenesis in vitro as a model to study cytophysiological aspects of zygotic embryogenesis in vivo are discussed.

Keywords: Triticum aestivum L., microspore, in vitro embryoidogenesis, in vivo embryogenesis, cell ultrastructure, reserve starch and lipids

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INTRODUCTION

In vitro androgenesis is an untraditional reproduction system having some parallels and analogues with other plant reproduction systems. The biological phenomenon of in vitro androgenesis consists in the switching of the morphogenetic developmental program of haploid anther cells from the usual gametophyte pathway (pollen grain formation) to the sporophyte pathway (haploid regenerant formation) under external stress conditions; in this case, cells realize their potential via various morphogenetic pathways in vitro (Kruglova et al., 2005; Segui-Simarro and Nuez, 2008; Segui-Simarro, 2010, 2016; Batygina et al., 2010; Batygina, 2011, 2014; Seldimirova and Kruglova, 2015, etc.). One of such pathways providing a sporophyte development of haploid anther cells is microspore embryoidogenesis representing the formation of embryoid (bipolar embryo-like structure) directly from an initial cell (strongly vacuolated microspore).

To date, various aspects of in vitro microspore embryoidogenesis are quite well studied in different plants, including wheat (Kruglova et al., 2005; Batygina et al., 2010; Segui-Simarro, 2010; Soriano et al., 2013; Seldimirova and Kruglova, 2015). The similarity in the passage through basic developmental stages was established between in vitro microspore embryoids and in vivo zygotic embryos of wheat (Seldimirova et al., 2004; Kruglova et al., 2005; Batygina et al., 2010). The detailed data on the ultrastructural analysis of wheat embryo development were obtained (HrSel et al., 1961; Smart and O'Brien, 1983; Chaban, 1986; Bannikova et al., 1991; Naumova and Matzk, 1998). At the same time, microspore embryoidogenesis was studied only at initial stages due to the revealing of a mechanism switching the developmental program of microspores from the gametophytic to sporophytic pathway; moreover, the studies were carried out mainly on other cereal species (Carreda et al., 2000;

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Ramirez et al., 2001; Testillano et al., 2002; González-Melendi et al., 2005; Góralski et al., 2005; Maraschin et al., 2005; Pulido et al., 2006), and only one study was devoted to wheat (Bonet and Olmedilla, 2000). In general, no detailed comparative analysis of a cell ultrastructure at all stages of microspore embryoidogenesis and zygotic embryogenesis of wheat was performed, though such analysis would make it possible to associate structural characteristics of cells with their physiological and biochemical status.

In this connection, the purpose of this study was a comparative ultrastructural analysis of in vitro and in vivo developing cells of microspore embryoids and zygotic embryos of wheat, respectively, using light microscopy (LM) and transmission electronic microscopy (TEM).

MATERIALS AND METHODS

The object of our study was a Fotos hybrid line of soft spring wheat (*Triticum aestivum* L.) characterized by a high frequency of in vitro embryoid formation in the anther culture. Donor plants were grown under field conditions at the research station of the Ufa Institute of Biology of the Russian Academy of Sciences (Ufa district).

Embryoids were obtained by the in vitro culturing of wheat anthers (Kruglova and Batygina, 2002) using Potato II induction medium (Chuang and Quyang, 1978) supplemented with kinetin (0.2 mg/L) and 2,4-D (0.1 mg/L) and then were cultivated under light until the appearance of seedlings. In vivo embryos were obtained by artificial pollination under field conditions.

To perform light microscopy, samples were fixed using the FAA mixture (Berlin and Miksche, 1976). Squash preparations stained by acetocarmine and permanent preparations stained by 1% iodine solved in 0.5% potassium iodide solution or by a periodic acid Schiff reaction were prepared according to Kruglova et al. (2013). Samples for the TEM examination were prepared according to the common recommendations (*Electron Microscopy...*, 1999). To do this, samples were fixed in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer, then postfixed in 1% solution of osmium tetroxide in the same buffer supplemented with sucrose (25 mg/mL), dehydrated by ethanol, acetone, and propylene oxide, and embedded in epoxy resin using an EMbed 812 embedding kit (EMS, United States). Ultrathin sections were obtained using a 8800 Ultrotome ultramicrotome (LKB Bromma, Sweden), contrasted with aqueous solutions of uranylacetate and lead citrate, and analyzed using a JEM-1200 EX electronic microscope (JEOL, Japan). The periodization of wheat embryogenesis (Batygina, 1987, 1997) and embryoidogenesis (Kruglova et al., 2005) and also the periodization of the wheat anther development (Kruglova, 1999) were used.

RESULTS

Ultrastructural Characteristics of in vitro Microspore Embryoids

At the moment of inoculation onto induction medium, anthers contained strongly vacuolated microspores (periodization: Kruglova, 1999) characterized by a clearly defined polarity: the nucleus was located opposite to the germ pore, and the large vacuole occupied the cell center (Fig. 1a).

According to data of the ultrastructural analysis, strongly vacuolated microspores were coated with the exine with well-defined layers (Fig. 1b). The nucleus contained nucleoli and condensed chromatin (Figs. 1a, 1b). The vacuole was electron-opaque and contained flaky substance (Figs. 1b, 1d, 1e). The cytoplasm was electron-dense due to a large number of both free and aggregated ribosomes (Figs. 1b–1e) and contained endoplasmic reticulum (ER) channels (Fig. 1c). Mitochondria were large, rounded, or oval and rarely had underdeveloped christas (Figs. 1c–1e). Plastids contained single thylakoids (Fig. 1e) and 1–3 starch grains (not shown). The cytoplasm also included low-activity cisternae of the Golgi complex (Fig. 1d), lipid drops, and various vesicles (not shown).

After 3 days of cultivation, some changes in microspore structure occurred. The nucleus, which gradually moved to a central position, contained a nucleolus with several vacuoles and condensed chromatin (Figs. 1f, 1g). The vacuole was fragmented due to the formation of cytoplasmic bands connecting perinuclear and peripheric cytoplasm (Figs. 1f, 1g). As a result, several small vacuoles were formed instead of the large one; these vacuoles were more or less uniformly distributed within the cell volume (Figs. 1g, 1i). These changes were accompanied by the loss of the polar organization by a microspore.

In general, ultrastructural characteristics of depolarized microspores agreed with those of strongly vacuolated polarized microspores. Their cytoplasm contained a large number of free and grouped ribosomes (Figs. 1h–1i), few short ER channels (Fig. 1h), and lipid drops (Fig. 1i). Internal membrane structures of mitochondria and plastids were weakly developed (Fig. 1h). Plastids contained a few small starch grains (Fig. 1g). In some cases, dividing plastids were observed (Fig. 1h).

Earlier, we showed that such microspores that had undergone a symmetrical mitosis (that is unusual for in vivo conditions) gave rise to embryoids, which, in turn, passed through the phases of blastomerization, organogenesis, and formed embryoid (Kruglova et al., 2005).

In the beginning of the blastomerization phase (5–7 days of cultivation), when the embryoid consists of 10–15 cells, these cells were still located within the exine (Fig. 2a). Internal cell walls of the embryoid were thin and have plasmodesm (Figs. 2b, 2d). Large

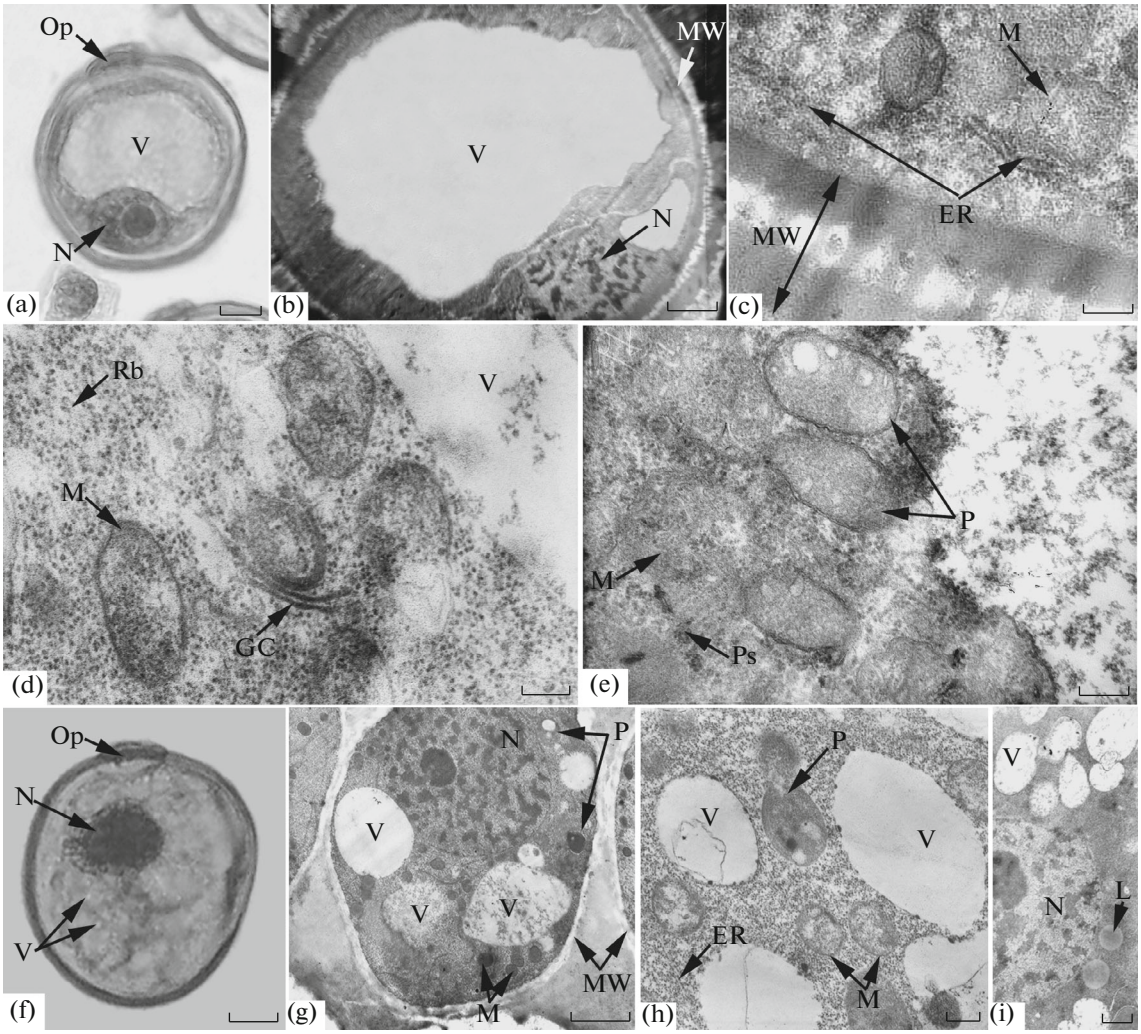

Fig. 1. Initial cell of the embryoid. (a) Initial cell: a strongly vacuolated microspore with a marked polarity. (b–e) Ultrastructural features of a strongly vacuolated microspore: (b) general view, (c–e) fragments of a cell structure. (f) Depolarized microspore. (g–i) Ultrastructural features of a depolarized microspore: (g) general view; (h, i) fragments of a cell structure. (a, f) Light microscopy, squash preparations. (b–e, g–i) Transmission electronic microscopy. V—vacuole; GC—Golgi complex; L—lipid drops; M—mitochondrion; Op—operculum; MW—microspore wall; P—plastid; Ps—polysome; Rb—ribosome; ER—endoplasmic reticulum; N—nucleus. Bar: (a, f, g) 10 μm, (b) 5 μm, (c–e) 200 nm, (h) 500 nm, (i) 1 μm.

nuclei (sometimes with several nucleoli) were located at the center of cells. Condensed chromatin was uniformly distributed within the nucleus (Figs. 2b, 2c). The nuclear envelope had some invaginations increasing the contact surface between the nucleus and cytoplasm (Fig. 2c). The cytoplasm was electron-dense and contained a large number of ribosomes often aggregated into dense conglomerations (Figs. 2d–2f) and a lot of vacuoles containing electron-dense flaky substance (Figs. 2b, 2c). Mitochondria were rounded and had well-developed cristae (Figs. 2d, 2e); in some cases, they were dumbbell-like and dividing (Fig. 2e). The number of plastid profiles increased, like the number of starch grains and their size (Figs. 2f–2h) that could be clearly visible in the case of a starch-specific histochemical staining of embryoid cells (Fig. 2a). Some of the starch-free plastids formed thylakoids (Figs. 2g, 2h). Sometimes dumbbell-like and dividing plastids were observed (Fig. 2g). The number of peripheral lipid drops increased (Figs. 2d–2f), and well-developed ER channels with dilatations were often observed between them (Fig. 2f). The Golgi complex was active and consisted of the stacks of vesicle-generating cisternae (not shown).

Due to active mitotic divisions, the size of an embryoid significantly increased after 12–15 days of cultivation, the transition to the globular stage occurred (Fig. 2i), and the embryoid leaved a microspore wall. Ultrastructural characteristics of such embryoid did not change as compared with the previous developmental stage (Fig. 2a), excepting a clear differentiation of two types of plastids: amyloplasts, which accumulate

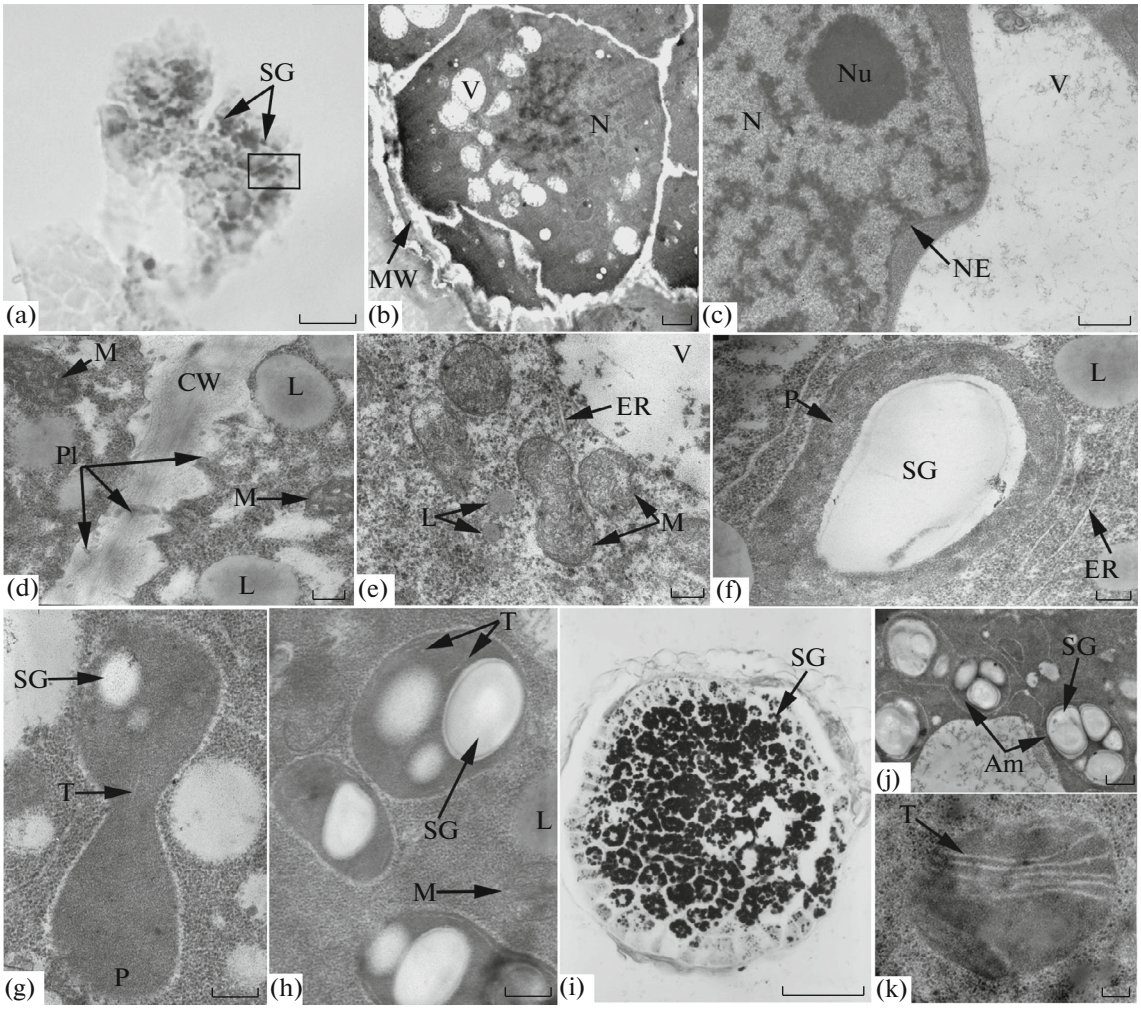

Fig. 2. Embryoid at the blastomerization phase and at the globular stage of development. (a) 15-cell embryoid. (b–h) Ultrastructural features of embryoid cells; (b) fragment of surface cells (indicated with a frame at the fragment (a)), (c–h) fragments of central cells. (i) Embryoid at the late globular stage. (j, k) Ultrastructural features of its central cells. (a, i) Light microscopy, permanent preparations, histochemical staining with iodine to detect starch. (b–h, j, k) Transmission electronic microscopy. Am—amyloplast; CW—cell wall; Pl—plasmodesm; T—thylakoid; Nu—nucleolus; NE—nuclear envelope, SG—starch grain. Other designations correspond to those of Fig. 1. Bar: (a) 25 μm, (b) 2 μm, (d–h, k) 200 nm, (i) 100 μm, (c, j) 1 μm.

starch in the form of large grains (Fig. 2j), and plastids with stacks of developing thylakoids, which probably represent juvenile chloroplasts (Fig. 2k).

In the course of further development, a transition to the organogenesis phase occurred at the 20th–21st days of cultivation. The embryoid polarity became clearly manifested via the segregation of the apical and basal poles (Fig. 3a). Cells of the apical and basal regions of the embryoid differed between themselves, especially in their ultrastructure.

Apical cells were meristematic and densely spaced; their large and rounded nuclei had invaginated envelopes and occupied the central position. Surface cells had thick outer walls (Fig. 3b) and did not differ from the central cells in their ultrastructure excepting the lower starch content (Figs. 3a, 3b). The cytoplasm of central cells contained ER channels associated with ribosomes and also many ribosomes, often in the form of conglomerations (Fig. 3c). Many lipid drops were located at the peripheral zone of cells (Fig. 3d). Plastids were represented mainly by amyloplasts filled with starch grains; some amyloplasts were very large and contained a large amount of starch (Fig. 3d). Plastids containing 1–2 starch grains and thylakoids were also observed (Fig. 3f). Multiple rounded or dumbbell-like mitochondria with well-developed cristae were usually observed in the regions with a high concentration of lipid drops (Fig. 3f).

Compared to apical cells, basal cells were more strongly vacuolated and formed intercellular spaces (Figs. 3a, 3g). A large number of lipid drops were located in both central (Fig. 3h) and peripheral regions of these cells (Figs. 3i, 3j). As in the case of apical cells, conglomerations of mitochondria with

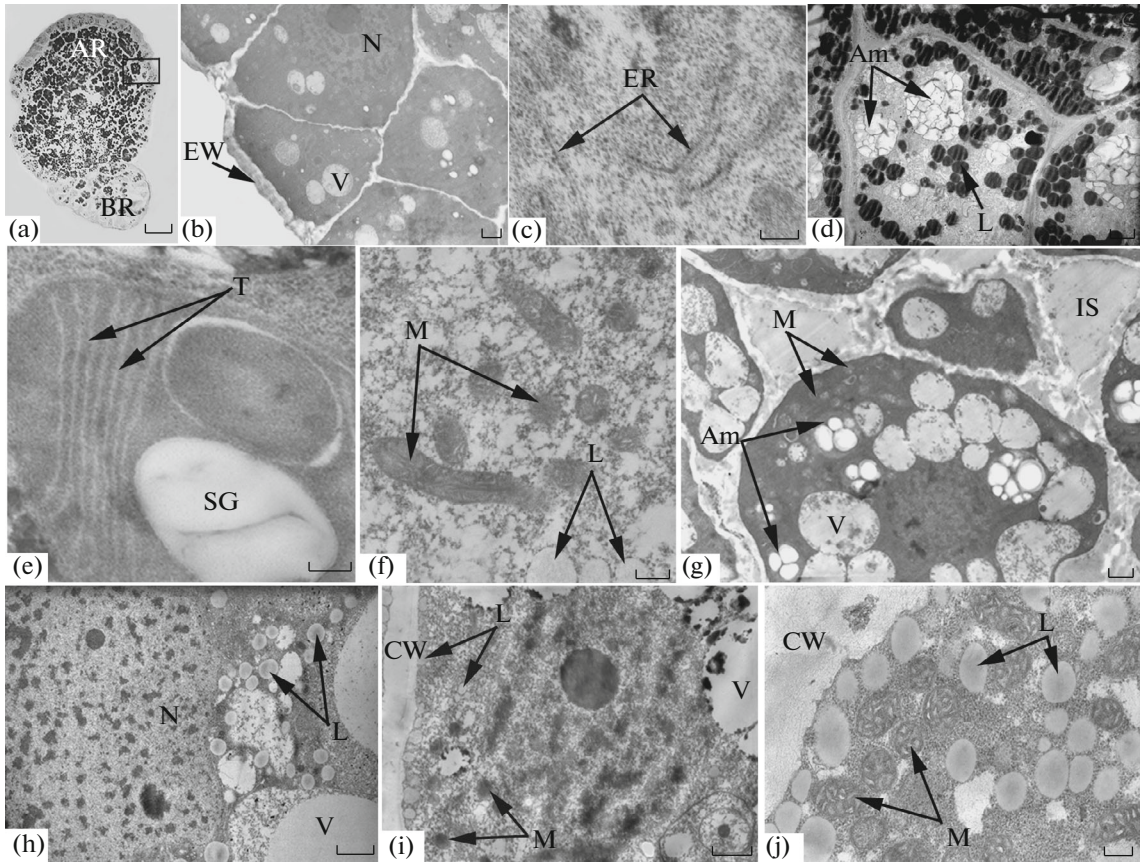

Fig. 3. Embryoid with the formed apical-basal axis (transition to the organogenesis phase). (a) General view. (b–j) Ultrastructural features of embryoid cells: (b) fragment of cells of the apical region near protoderma, at the border of the initiating scutellum and shoot meristem (indicated with a frame at the fragment (a)), (c–f) fragments of cells located in the center of the apical region, (g–j) fragment of cells of the basal region. (a) Light microscopy, permanent preparations, histochemical staining with iodine to detect starch. (b–j) Transmission electronic microscopy. AR—apical region; BR—basal region; EW—external wall; IS—intercellular space. Other designations correspond to those of Figs. 1 and 2. Bar: (a) 50 μm, (b, d) 2 μm, (c, f, g, j) 200 nm, (e) 100 nm, (h, i) 1 μm.

well-developed internal structures (Fig. 3j) were observed among peripheral lipid drops. Plastids were represented by starch-containing amyloplasts (Fig. 3g).

In the beginning of the organogenesis phase (25–27 days of cultivation), due the meristematic origin of emryoids, the majority of their ultrastructural characteristics coincided with those of the earlier stages (Fig. 4a); however, there were also some differences. For example, the number of amyloplasts and starch grains in the cells of shoot and root meristems was reduced (Figs. 4b, 4c). Shoot meristem cells had well-developed chloroplasts with thylakoids arranged into grana (Fig. 4d). Surface cells of scutellum became elongated, but hardly differed from other cells. Both surface and other cells had a large number of small vacuoles, some of which contained electron-dense inclusions (Fig. 4e). The cells contained many ER cisternae, lipid drops, and amyloplasts with large starch grains (Figs. 4e, 4f). Cells of the basal part of the embryoid were arranged loosely and were partially destructed; their amyloplasts contained single small starch grains, and a large number of lipid drops was observed in the whole cell volume (Fig. 4g).

The content of starch and lipids in formed embryoids (40–42 days of cultivation; Fig. 4h) remained unchanged; the shoot apexes and leaf primordia contained well-developed chloroplasts (not shown).

Ultrastructural characteristics of in vivo zygotic embryos. In 12 h after the artificial pollination, a typical pear-shaped zygote (Fig. 5a) was observed in the embryo sac of a wheat ovule. A large nucleus with condensed chromatin and a nucleolus with several vacuoles were observed in the apical part of a cell (Figs. 5a, 5b). A large number of vacuoles with electron-dense inclusions were located around the nucleus (Fig. 5b). Perinuclear space contained multiple organoids represented by medium-sized rounded mitochondria with poorly developed cristae (Figs. 5b, 5c) and by amyloplasts containing 1–3 starch grains (Figs. 5b, 5d). Both free and aggregated ribosomes were observed in the cytoplasm (Figs. 5c, 5e). ER was granular and

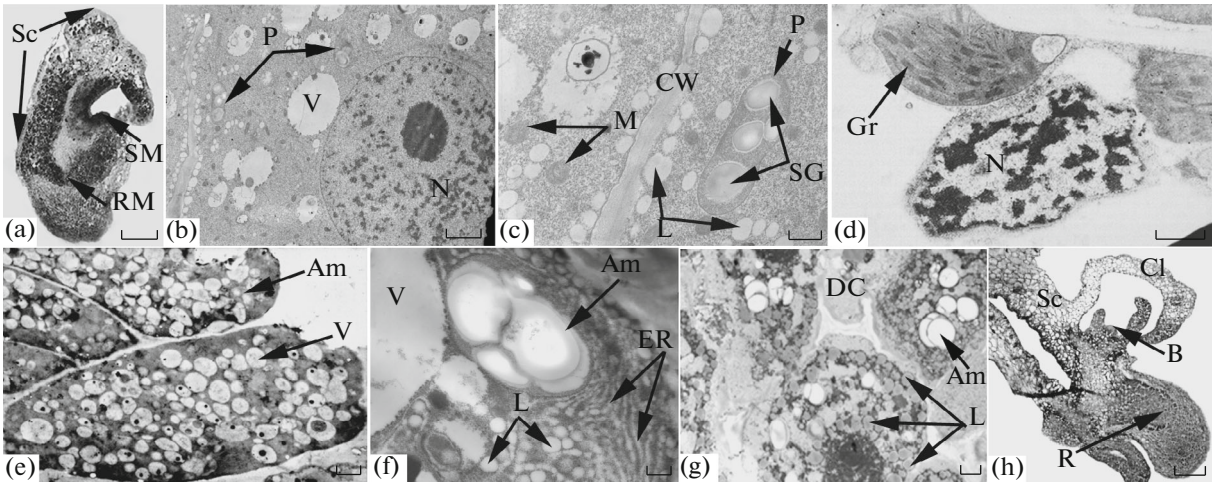

Fig. 4. Embryoid at the organogenesis phase and formed embryoid. (a) General view at the organogenesis phase. (b–g) Ultrastructural features of embryoid cells: (b–d) fragments of cells located in the region of the apical shoot meristem, (e–f) fragments of scutellum surface cells, (g–h) cells of the basal part; (h) general view of a formed embryoid. (a, b) Light microscopy, permanent preparations, histochemical staining with iodine to detect starch. (b–g) Transmission electronic microscopy. SM—shoot meristem; Gr—grana; DC—degenerating cell; RM—root meristem; Cl—coleoptile; R—root; B—bud; Sc—scutellum. Other designations correspond to those of Figs. 1–3. Bar: (a) 100 μm, (b, g) 2 μm, (c, f) 500 nm, (d) 200 nm, (h) 500 μm.

included elongated, sometimes tortuous and dilated channels; the Golgi complex was represented by the stacks of vesicle-generating dictyosomes (Fig. 5e). The cytoplasm contained lipid drops, which were usually aggregated and associated with elongated ER channels (Fig. 5d).

One day after the pollination, the zygote divided and formed a two-cell embryo. From this time, the blastomerization phase began. The further divisions resulted in the formation of a multicellular embryo with a differentiated suspensor (Fig. 5f).

The cells of the embryo and suspensor differed in their ultrastructure. Both surface and central cells of the apical part of the embryo had similar characteristics; the only difference was that surface cells had thicker outer walls, like in the case of the embryoid at the same stage of development (Fig. 5f). Both groups of cells had cytoplasm containing many organoids and large central nucleus often with invaginations of the nuclear envelope (Fig. 5g). Cell walls (excepting outer ones) contained plasmodesms (Fig. 5h). Cells were less vacuolated than those of a zygote (see Figs. 5g, 5b). Many vacuoles were characterized by a diffusive distribution of the electron-dense material and sometimes contained flake-like inclusions (Fig. 5g). The cytoplasm contained a lot of free ribosomes, partially in relatively large and dense conglomerations (Figs. 5h, 5j). The number of mitochondria increased, and their cristae became more developed (Figs. 5g, 5i). Plastids had single lamellae; the size and the number of starch grains was significantly reduced compared to the zygote (see Figs. 5i, 5d). Some plastids began to form stacks of thylakoids that probably evidenced to their transformation into juvenile chloroplasts (Fig. 5j). The ER proliferation was observed in the form of the increasing length and number of channels (Figs. 5h–5j). The Golgi complex was active and consisted of the group of curved vesicle-generating dictyosomes. Some of the dictyosomes also increased in their size (Fig. 5i). The cytoplasm of embryo cells hardly contained lipid drops.

Cells of the suspensor located in the basal part of the embryo were more vacuolated than apical cells. Many vacuoles contained inclusions resembling degenerated mitochondria and plastids (Fig. 5k). The cytoplasm contained a large number of amyloplasts with 1–2 small starch grains (Fig. 5l) and numerous single or aggregated lipid drops (Figs. 5l, 5m). The number of mitochondria at the places of concentration of lipid drops was rather small (Fig. 5m).

Since the initiation of the scutellum and shoot apex (Fig. 6a), the embryo passed to the organogenesis phase. In the beginning of this stage (7–9 days after pollination), shoot meristem cells were characterized by a large central nucleus with uniformly distributed condensed chromatin and 1–2 nucleoli (Fig. 6b). The nuclear envelope had invaginations (Figs. 6b, 6c). The cytoplasm contained some small vacuoles with electron-dense vesicular inclusions (Fig. 6b) and a large number of free ribosomes. Due to the disaggregation of ribosomal groups, their number decreased (Fig. 6d). Again, small amyloplasts with 1–3 starch grains were observed in the cytoplasm (Fig. 6b), along with juvenile chloroplasts, whose number and level of development were comparable with the preceding developmental stage (Fig. 6d). Mitochondria became more juvenile than those at the blastomerization phase (Figs. 6c, 6d). Long ER channels often formed con-

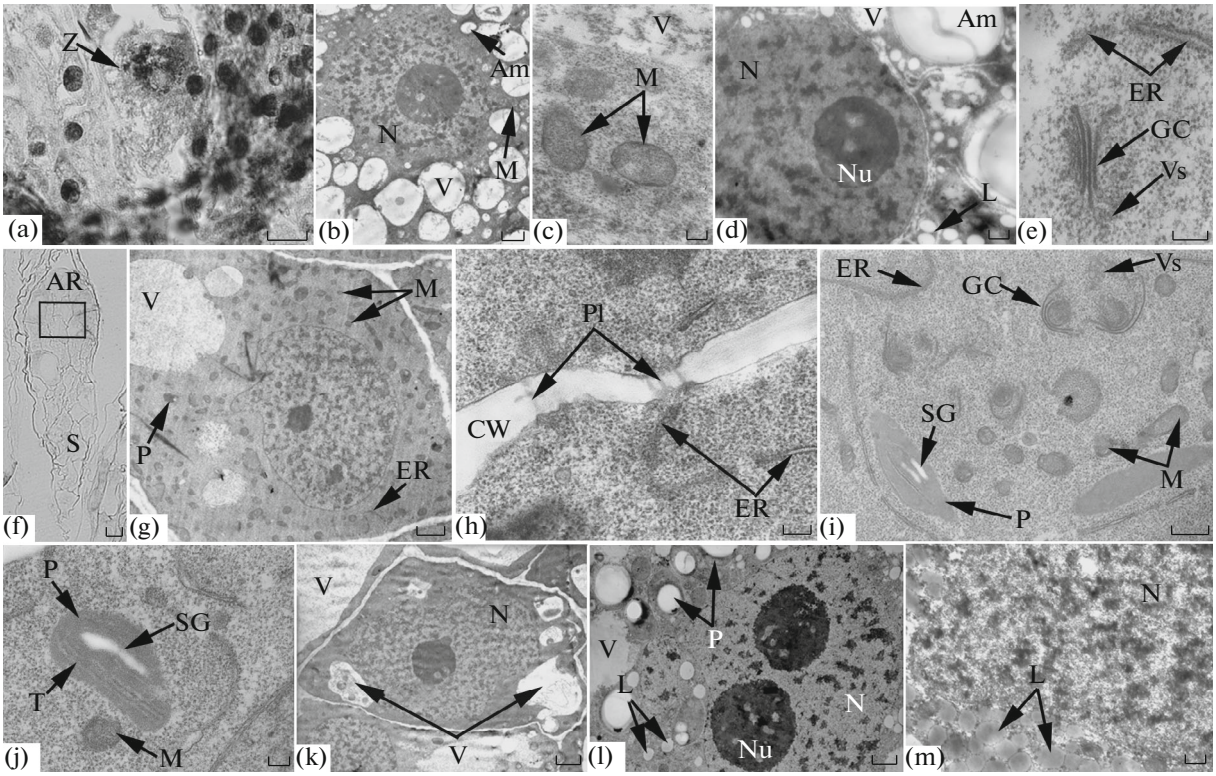

Fig. 5. Zygote and embryo at the blastomerization phase. (a) General view of a zygote. (b–e) Ultrastructural features of zygotic cells near the nucleus and adjacent cytoplasm. (f) General view of an embryo. (g–j) Ultrastructural features of central cells of the apical part of embryo (indicated with a frame at the fragment (a)). (k–m) Ultrastructural features of the cells of the basal part of embryo (near the suspensor). (a, f) Light microscopy, permanent preparations, histochemical staining with (a) Schiff–periodic acid and (f) iodine to detect starch. (b–e, g–m) Transmission electronic microscopy. Vs—vesicle of the Golgi complex; Z—zygote; S—suspensor. Other designations correspond to those of Figs. 1–4. Bar: (a) 20 μm, (b, g, k) 2 μm, (c, e, h, j) 200 nm, (d, i, m) 500 nm, (f) 10 μm, (l) 1 μm.

centric systems (Fig. 6c). The Golgi complex was active and was positioned along cell walls (Fig. 6d). Single lipid drops were observed in the cytoplasm (Fig. 6c).

Cells of the central part of the scutellum were strongly vacuolated. The content of their small vacuoles was diffuse (Fig. 6e), like in the cells of the central part of embryoid at the same stage of development. The cytoplasm contained a large number of Golgi cisternae with vesicles (Fig. 6f). Plastids contained starch grains; mitochondria were characterized by well-developed cristae (Fig. 6f). Long ER channels were observed at the peripheral part of cells; they were associated with numerous lipid drops located along the plasmalemma (Figs. 6f, 6g).

Suspensor cells were also strongly vacuolated and had clear manifestations of autolysis that initiated their destruction (Fig. 6h).

In the forming embryo (15–17 days after pollination, Fig. 6i), scutellum cells contained larger starch-accumulating amyloplasts (Fig. 6j), than at the preceding stage. Single chloroplasts from the cells of the shoot meristem became well developed (Fig. 6k).

In the morphologically mature embryo (21–23 days after pollination, Fig. 6l) the number of ER channels in bud cells was significantly reduced (Fig. 6m). Ribosome conglomerates disappeared in cells of all organs (see, for example, Fig. 6m).

DISCUSSION

According to the data of ultrastructural analysis, the processes of in vitro microspore embryoidogenesis and in vivo zygotic embryogenesis of wheat are characterized by a certain similarity.

One of such similar features is a high metabolic activity of initial cells of both embryoids and embryos. At the early stages of in vitro cultivation, strongly vacuolated microspores and depolarized microspores are characterized by a synthetic activity. The nuclear envelope often has invaginations, which increase the contact surface between the nucleus and cytoplasm. The observed contact between the nuclear envelope and condensed chromatin evidences to the functioning of a near-membrane chromatin, which is considered to be connected with the initiation of replication and, in some cases, DNA transcription (Chentsov,

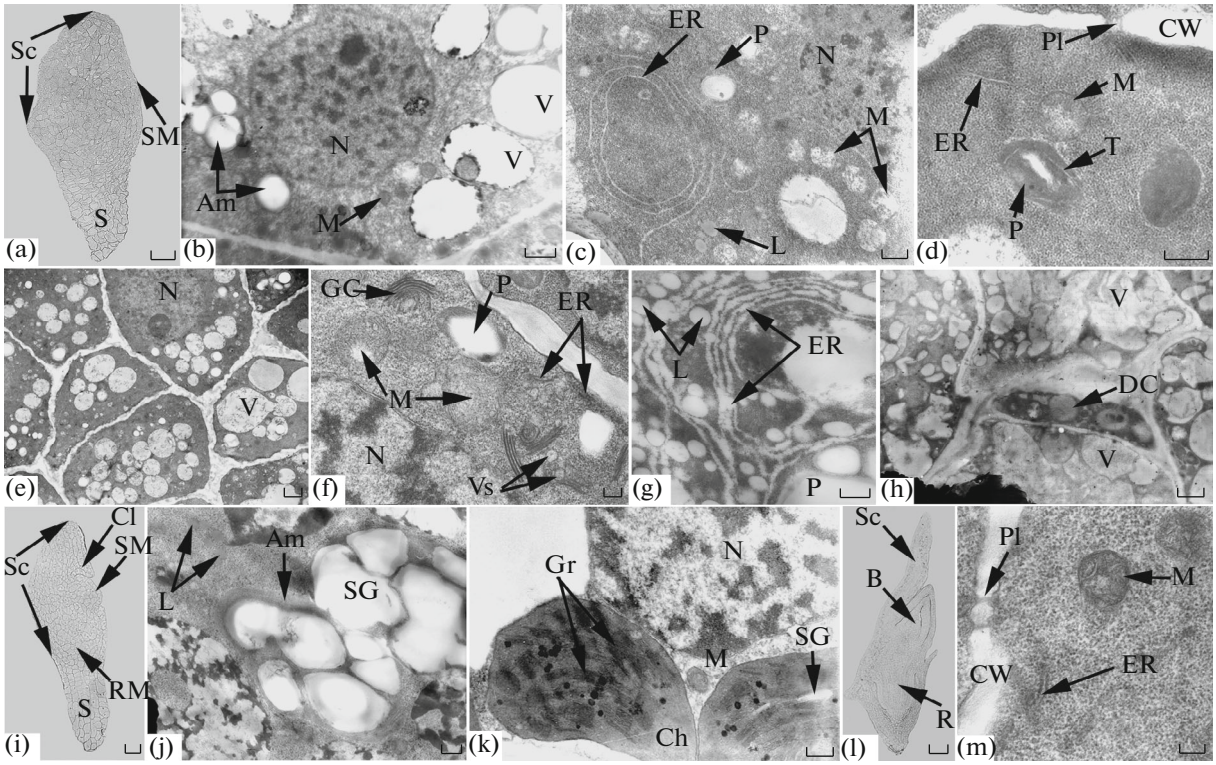

Fig. 6. Embryo at the organogenesis phase and the formed embryo. (a) General view in the beginning of the organogenesis phase. (b–h) Ultrastructural features of embryo cells at the (b–d) region of the forming shoot meristem, (e–g) subepidermal scutellum cells, and (h) suspensor. (i) General view of the embryo at the end of the organogenesis phase. (j) Ultrastructural features of subepidermal scutellum cells. (k) Mature chloroplast in the shoot meristem cell at the end of the organogenesis phase. (l) General view of a formed embryo. (m) Ultrastructural features of the shoot meristem cell of a bud. (a, i, l) Light microscopy, permanent preparations, histochemical staining with iodine to detect starch. (b–h, j, k, m) Transmission electronic microscopy. Ch—chloroplast. Other designations correspond to those of Figs. 1–5. Bar: (a) 25 μm, (b–d, j) 500 nm, (e, h) 2 μm, (f, g, k, m) 200 nm, (i) 50 μm, (l) 100 μm.

2004). Similar data on the ultrastructure of strongly vacuolated and depolarized microspores and cells of early-stage embryoids located within the exine were obtained for maize (Barnabas et al., 1987; Góralski et al., 2005) and wheat (Bonet and Olmedilla, 2000; Carreda et al., 2000; Maraschin et al., 2005). However, it was shown that the number of ribosomes in the cytoplasm of either initial wheat microspores or microspores that had undergone the first symmetrical division decreases, which indicates a decreased intensity of the protein and mRNA synthesis after the switching of the developmental program of these cells from gametophytic to sporophytic pathway (Bonet and Olmedilla, 2000). In general, the question on the dependence of the induction of a sporophytic program of development on the metabolic activity of initial microspores requires the further investigation.

The similar ultrastructural characteristics indicating high metabolic activity were also observed for a zygote, an initial cell of a wheat embryo.

In the beginning of the blastomerization phase, the wheat embryoid still remained within exine, which became thickened; a similar observation was also made for the embryoids of maize (Barnabas et al., 1987) and wheat (Bonet and Olmedilla, 2000).

In addition, internal cell walls of both wheat embryoids and embryos are characterized at this stage of development by the presence of plasmodesms. This fact can be easily explained, since plasmodesms provide a close connection between cells and mediate the transfer of factors influencing on the cell growth, division, and differentiation (*Plasmodesmata*..., 1999).

Like initial cells, both embryoid and embryo cells are characterized by a high metabolic activity at the blastomerization phase and in the beginning of the organogenesis phase; this activity is required for the synthesis of constitutional compounds during reproducing divisions increasing the number of embryoid/embryo cells. This fact is confirmed by the presence of well-developed mitochondria and plastids in these cells. In addition, the visible number and length of ER cisternae in the cytoplasm of both cell types increases. The agranular ER is usually located between lipid drops and is probably involved in their synthesis. The proliferation of agranular ER is connected with the synthesis of materials required for the cell wall formation (Bannikova et al., 1991). The Golgi complex is also involved in this

process; its activity in embryoid and embryo cells increases and is manifested via the appearance of vesicles segregating from the Golgi cisternae. The presence of granular ER cisternae and an increased number of polysomes are considered to be connected with the active protein biosynthesis (Chentsov, 2004). The increase in the number of mitochondria, complication of their internal membrane structure, and localization near lipid drops, observed for both types of cells at these stages of development, probably indicate an increased energy consumption of cells that has been already shown for other plants and organs (Chentsov, 2004).

During the blastomerization phase, cells of wheat embryoids and embryos were characterized by a large number of vacuoles. Similar data were obtained by other authors, who studied early developmental stages of maize embryoids and considered that vacuoles, due to the presence of various inclusions, provide the excretion of cell catabolites (Barnabas et al., 1987). Probably, vacuoles of wheat embryoids and embryos also have the same function during the blastomerization phase.

Starting from the organogenesis stage, vacuoles remained only in the cells of the basal part of embryoids and in the suspensor cells of embryos. According to the opinion of some authors, vacuoles in the suspensor cells may function as autolytic compartments participating in the destruction of this organ (Taylor and Vasil, 1995). It is quite possible that vacuoles of wheat embryoids and embryos play the same role.

At the end of the organogenesis phase, shoot meristem cells of both embryoids and embryos contained well-developed chloroplasts. Therefore, embryoids and embryos of the studied wheat variety may be described as chlorophyll bearing, and wheat may be referred to the group of chloroembryophytes, i.e., plants whose embryos (and, as we consider, embryoids) contain chlorophyll (Zhukova, 1992; Puthur et al., 2013). The phenomenon of in vivo chlorophyll-bearing embryos is widely observed in flowering plants, but, according to Zhukova (1997), has not been ever described for wheat. The specificity of the "embryonic" photosynthesis considers it is directed mainly on the accumulation of nutrient reserves in the forming seeds, while ATP and NAD(P) · H synthesized in light reactions are used mainly to transform sucrose received from the maternal plant to fatty acids (Smolikova and Medvedev, 2016). Obviously, in the case of light-cultivated embryoids, the photosynthetic process is directed to the same purpose, and the synthesized ATP and NAD(P) · H are consumed to provide the biochemical transformation of sucrose from the nutrition medium. At the same time, in contrast to embryoids, we did not observe a clear green color of the embryo bud at any stage of development, probably due to the singleness of chloroplasts in shoot meristem cells.

Along with a significant similarity between the ultrastructural characteristics of the cells of wheat embryoids and embryos, some differences were also observed.

While the above-described ultrastructural characteristics remained unchanged in embryoid cells during the whole period of their development, the length of ER cisternae in embryo cells significantly decreased to the end of the organogenesis phase. This phenomenon is probably connected with the preparation of the embryo to the dormant period, which is absent in the case of in vitro embryoid development. The same reason probably determined an increased number of free ribosomes and the juvenilization of mitochondria in embryo cells.

The next distinction is that the number of lipid drops presented in embryoid cells and usually positioned along the cell wall significantly exceeded that of embryo cells. Some authors reported about a significant reduction of the number of lipid drops in embryo cells to the beginning of the organogenesis phase and about an increase in their number in the scutellum cells of mature embryo prior the transition to the dormancy period (Bannikova et al., 1991; Taylor and Vasil, 1995). This fact corresponds to the idea that the use of initial lipid reserve as the energy source at the certain stages of embryo development and their further accumulation as storage compounds are typical for the embryogenesis of angiosperms (Bannikova et al., 1991; Naumova, 1997; Raghavan, 1997).

A high lipid content in wheat embryoid cells was observed in the whole course of their development, starting from the organogenesis phase. According to some researchers, a large number of lipid drops was also observed in the cells of globular embryoids of wheat (Bonet and Olmedilla, 2000) and maize (Tsay et al., 1986; Barnabas et al., 1987; Góralski et al., 2005). In general, the presence of lipids seems to be a common typical feature of developing microspore embryoids of cereals. The ability for the synthesis and utilization of lipids may be caused by the switching of the initial microspore development program from the gametophytic to sporophytic pathway (Tsay et al., 1986; Barnabas et al., 1987). There is also an opinion that lipid accumulation is caused rather by programmed degradation of the tapetal layer of the anther wall or microspore membranes than by their de novo synthesis (Barnabas et al., 1987). In our case, the presence of elongated agranular ER channels among lipid drops made it possible to suppose that this organoid intensively synthesizes lipids consumed by actively dividing embryo cells. This hypothesis was evidenced by the presence of mitochondria with well-developed cristae near the groups of lipid drops. Based on these facts, one may consider lipids as an alternative source of energy required for active cell divisions during the formation and subsequent germination of embryoids in the absence of the endosperm.

Another difference between embryoid and embryo cells was the dynamics of the starch synthesis during

the development process. In early embryos, the plastidome was usually represented by small amyloplasts with single small starch grains; starch accumulation in scutellum cells started only at the end of the organogenesis phase. At the same time, starch accumulation in embryoid cells started already at the stage of the initial cell (microspore); the amount of starch visually increased in all organs and tissues as the embryoid continued to develop. Similar data were obtained during the study of initial cells of maize (Barnabas et al., 1987; Góralski et al., 2005) and wheat (Bonet and Olmedilla, 2000; Carreda et al., 2000) embryoids.

According to the opinion of many authors, embryoids are developed from microspores with a high starch content, since starch provides cell divisions with energy (Dunwell and Sunderland, 1974; Barnabas et al., 1987; Carreda et al., 2000). However, Carreda et al. (2000) showed the existence of two plastid-development programs in microspores of barley. The first program results in the plastid differentiation via the amyloplast way: they intensively accumulate starch and lose thylakoids and ability for divisions, i.e., follow the irreversible gametophytic program of development. According to the second program, in the beginning of cultivation, plastids lose starch and are able to turn to chloroplasts, i.e., they are still capable of differentiation and development via the sporophytic program. Note that, in both cases, microspores realize a sporophytic program of development resulting in the formation of embryoids and then regenerants (albinos or green, depending on the program).

The presence of a large amount of starch in cells of the studied wheat embryoids was also observed at the later stages of their development that corresponded to the earlier published data for wheat and other flowering plants (Rahman, 1993; Reynolds, 1993; Hause et al., 1994; Yeung et al., 1996; Palmer and Keller, 1997; Seldimirova, 2013).

Sucrose is often used as an osmoregulator and carbon source in nutrient media intended to obtain microspore embryoids (Dunwell, 2010, etc.). Sucrose is easily absorbed by cells, since its molecules are able to pass through cell membranes in an intact form using specific carriers. The absorbed sucrose may either be stored in vacuoles or be converted to starch and stored in plastids (Ilic-Grubor et al., 1998).

It is known that the sucrose conversion to starch is controlled by several enzymes. One of the key enzymes is sucrose synthase (SUS, EC 2.4.1.13) (Dai, 2010). According to some data, indolyl-3-acetic acid may directly activate sucrose synthase (Turkina and Sokolova, 1972). Induction medium intended for in vitro microspore embryoidogenesis of wheat includes 2,4-dichlorophenoxyacetic acid, a synthetic analogue of indolyl-3-acetic acid. It is quite possible that this synthetic auxin may influence on sucrose synthase in the same way as indolyl-3-acetic acid.

According to some authors, starch accumulation in embryoid cells is caused by the excess sucrose concentration in nutrient medium (Rahman, 1993; Yeung et al., 1996; Ilic-Grubor et al., 1998b). Such redundancy should influence on the cell content and the tissue structure of embryoids. This opinion was confirmed by the fact that zygotic embryos of rape, extracted from the ovule at the torpedo stage and cultivated in vitro for 2 weeks on sucrose-containing medium, accumulated starch grains in the same way as microspore embryoids (Rahman, 1993).

To eliminate starch accumulation as a probable result of a sucrose influence, an attempt to cultivate rape microspores on the medium containing a high concentration of polyethylene glycol as the osmoregulator and the medium with limited content of sucrose as the carbohydrate source was made (Ilic-Grubor et al., 1998). The morphology of embryoids cultivated on the first medium was similar to that of zygotic embryos at the corresponding stage of development. Embryoids cultivated on the second medium differed from zygotic embryos by their size, color, and cotyledon structure; in addition, they accumulated starch that confirmed the dependence of this process on the external conditions.

However, in vitro study of the somatic embryoidogenesis in two spruce species showed the variation of carbohydrate types and their amount in nutrient medium did not influence on the concentration of endogenous starch in embryoids (Iraqi and Tremblay, 2001). In addition, the lack of the effect of a sucrose concentration in nutrient medium on the starch content in cells of micropropagated hosta explants was reported (Gollagunta et al., 2004). Therefore, this question requires further studies.

In vivo embryos obtain sucrose from surrounding maternal tissues and use it, along with other compounds, for respiration and formation of the embryo body. The amount of consumed sucrose is determined by ripening seeds, which represent strong attracting centers. The state of these centers determines the volume of seed demand for sucrose, which is provided by the photosynthesis. If external conditions do not limit the photosynthetic process, then the main role in its determination belongs to the processes of formation and growth of new structures (Medvedev, 2004). Carbohydrates also represent the main energy source for cell divisions in the embryo, and amylolytic products are used for the respiration and various synthetic processes occurring during seed germination (Naumova, 1997). In general, physiological mechanisms, which provide cell extension during germination, function only in the case of a proper energy provision and uptake of storage compounds from their own reserves (Obrucheva and Antipova, 1997; Liu et al., 2006). Therefore, in our opinion, one should not exclude the possibility that the starch accumulation in embryoids occurring since the early stages of their development is

intended to form an energy resource that can be rapidly demanded by a microspore embryoid during the proper stage of its in vitro development.

It is known that endosperm represents an in vivo energy source for a developing embryo. In the case of an embryoid, which is formed at the absence of the endosperm, some of its cells probably assume this function. This hypothesis agrees with the data of some other authors (Testillano et al., 2002; Maraschin et al., 2005; Pulido et al., 2006), according to which maize embryoids consist of two domains, embryonal and endosperm-like, which differ in their size, cell structure, gene expression patterns, and functions (endosperm-like domain has a storage function). Maize embryoids contain genes characterized by specific spatial and temporal expression patterns during early zygotic embryogenesis. These genes include ESR, ZmAE1, ZmAE3 (all are expressed at the micropylar end of the endosperm), OCL3 (expressed in the suspensor), and LTP2 (expressed in the protoderm); genes specific for the endospersm and the embryo itself were expressed in different parts of embryoids (Magnard et al., 2000; Testillano et al., 2002; Massonneau et al., 2005). Similar data were obtained by the histological study of early embryoids of other cereal species; these embryoids also consisted of two domains differing in the structural characteristics of their cells (Bonet and Olmedilla, 2000; Ramirez et al., 2001; Góralski et al., 2005; Maraschin et al., 2005; Dubas et al., 2010). Moreover, the study of early barley embryoids obtained from the in vitro culture of isolated microspores revealed hordeins, basic storage proteins of the endosperm (Pulido et al., 2006). This fact also confirms that embryoids include cell groups, which, like endosperm, accumulate starch to provide an energy resource.

Thus, in vitro microsporial embryoidogenesis should be considered as the determinated and plastic process intended to form a normal regenerant plant. The plasticity is manifested via the use of alternative metabolic pathways for the creation of energy resources providing the growth and development of an embryoid at the absence of the endosperm. These processes may be also considered as an example of a polyvariant solution of problems providing an improvement of the reliability system of the organism (Grodzinskiy, 1983; Batygin, 1986; Veselovskiy, 1987; Batygina, 2012, 2014).

The similarity between the in vitro development of microspore embryoids and in vivo development of zygotic embryos, including the similarity of ultrastructural characteristics of their cells, confirm the concept based on the systemic approach and proposed by T.B. Batygina (1987, 1997, 2014); this concept considers the universality of the in vivo, in situ, and in vitro morphogenetic processes of plants irrespective of the type of their reproductive structure, way of reproduction, and growing conditions. According to the author's opinion, such universality should be manifested starting from the structure of initial cells of different reproductive structures. In fact, according to the data of light microscopy and ultrastructure analysis, both strongly vacuolated microspore (initial cell for in vitro embryoidogenesis) and zygote (initial cell for in vivo embryogenesis) are characterized by the polarity of their structure that means the presence of functionally important asymmetric structures generating in response to vectorized internal or external stimuli (Medvedev and Sharova, 2011). In the course of the further development, such polarity determined a gradual formation of the apical-basal organization of both embryoids and embryos along with the differentiation of apical and basal poles and shoot and root meristems. Moreover, both embryoids and embryos are characterized by the cell and histological ("histogenous") differentiation. Embryoids and embryos pass through the same developmental phases: initial cell–blastomerization–organogenesis–formed structure. They generate identical organs, whose formation, in general, is similar concerning the sequence and way of their initiation. Starting from the initial cell and in the course of the whole process of development of embryoids and embryos, an obvious similarity was revealed in the ultrastructural characteristics of their cells.

In spite of some specific features, such universality makes it possible to propose the use of in vitro developing microspore embryoids as a model to study the developmental cytophysiology of in vivo zygotic embryos.

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