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CYTOLOGICAL FEATURES OF VARIOUS MICROSPORE DERIVATIVES APPEARING DURING CULTURE OF ISOLATED MAIZE MICROSPORES

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Microspore derivatives occurring in culture of maize microspores were studied by light and electron microscopy. The cultures showed a high degree of heterogeneity throughout the whole period of cultivation. Several types of androgenic structures at different developmental stages were observed, indicating a high level of asynchrony among microspores following the androgenic pathway. During the first 5 to 7 days of culture, multicellular structures developed inside the exine. At later stages of culture the developing microspores ruptured the pollen wall and formed structures variable in cellular characteristics. Three main types were distinguished: (i) one-domain structures consisting of small or large cells only; (ii) two-domain structures with large and small cells, the domains differing in size and cellular characteristics; (iii) callus-like structures. The two-domain structures seem to resemble zygotic proembryos which also contain two different parts, the suspensor and the apical region. The observed variability is most likely related to differences in gene activity during the inductive stage of androgenesis and the genetic properties of the microspores themselves, rather than to the conditions of in vitro culture.

Key words: *Zea mays* L., maize, microspore embryogenesis, androgenesis, cytological analysis, ultrastructure.

INTRODUCTION

Androgenesis, also called microspore or pollen embryogenesis, is one of the most striking examples of cellular totipotency in plants. Unicellular microspores, or pollen at the early bicellular stage, are able to switch the developmental pathway irreversibly to form multicellular structures and finally mature plants with a haploid or dihaploid number of chromosomes.

Androgenic development was first demonstrated by Guha and Maheshwari (1964, 1966) in anther culture of *Datura innoxia*. Since that study it has been reported in over 200 species of angiosperms belonging to more than 50 genera and 25 families of dicots and monocots. Numerous factors have been described as affecting the induction of androgenesis. The most important are the genotype of the donor plant, the stage of pollen development, the composition of the nutrient medium, pretreatment of flower buds or inductive treatment of isolated microspores, and the physiological state and conditions of growth of donor plants (for review see: Jähne and Lörz, 1995; Góralski et al., 1999; Indrianto et al., 1999; Smýkal, 2000).

Embryogenesis from isolated microspores has been studied by light and electron microscopy to find cytological markers for the embryogenic state of the microspore/pollen (for review see: Rodríguez-Garcia et al., 2000; Testillano et al., 2000). *Brassica napus* and *Nicotiana tabacum* are the two principal model systems in which microspore embryogenesis has been studied in detail; androgenesis can be easily and efficiently induced in these plants.

Cytological studies have yielded many insights into the early events of androgenesis, but they have focused mainly on early events in the development of androgenic structures. The developmental sequence from individual microspore to whole plant has been followed only in rape (Hause et al., 1994) and wheat

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(Touraev et al., 2000). Pioneer cytological work was done by Sunderland (1973). Further observations of pollen-derived structures in several species indicated that the sporophytic development of microspores can be assigned to five basic pathways, based mainly on the types of nuclear (and cell) divisions and their later fate in growing microspores (for review see: Góralski et al., 1999).

In maize, an efficient protocol for culture of isolated microspores was developed by Gaillard et al. (1991). In this species, microspore cultures are very heterogeneous, and several types of embryogenic structures at different developmental stages can be observed (Barnabás et al., 1987; Pretova et al., 1993; Góralski et al., 1999; Alché et al., 2000; Magnard et al., 2000; Testillano et al., 2000, 2002).

The variability of androgenic structures occurring in maize microspore culture is incompletely understood. Most data deal with young androgenic structures (5 to 7 days old). The older stages of embryogenic development are much less studied.

The objective of this paper is to analyze, by light and electron microscopy, the cytological features of microspore derivatives appearing in maize microspore culture during 30 days of cultivation.

MATERIALS AND METHODS

MICROSPORE CULTURE

The experiments were carried out on the androgenic maize hybrid DH5×DH7 (Barloy et al., 1989). Before microspore isolation, tassels were pretreated for 7 to 21 days at 7˚C. Microspores were isolated according to the protocol described previously (Gaillard et al., 1991; Góralski et al., 2002). The androgenic structures used in this study originated from cultures in 4 cm Petri dishes containing 4 ml microspore suspension in liquid medium supplemented with sucrose (S120). The cultures were kept in the dark at 28˚C (for details of the procedure see: Góralski et al., 2002).

CYTOLOGICAL STUDIES

Material for light microscopy

The observations used freshly isolated microspores (day 0 of culture), multicellular structures enclosed in exine (days 5 to 7 of culture), structures released from the pollen wall (days 12 to 14 of culture), and macroscopic structures (day 30 of culture). The study included analysis of whole structures under visible light and UV (DAPI staining), and semithin sections.

For nuclei staining, 0.5 ml culture suspension was transferred to an eppendorf tube, washed in distilled water and centrifuged (65 g, 3 min). The pellet was resuspended in 1 ml mixture containing DAPI (5 µg ml^{-1}), pH 4.0 citrate/phosphate (0.1/0.2 M) buffer and Triton X100 (10 µl ml^{-1}). That suspension was kept in darkness for at least 1 h, then mixed and observed under UV with a Nikon Optiphot-2 microscope.

For histological study the material was fixed in 5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 2 h at room temperature. After dehydration in an ethanol series, the samples were embedded in Technovit 7100 (2-hydroxyethyl-metacrylate) (Heraeus Kulzer). The sections (4 µm thick) were stained with 1% aqueous solution of toluidine blue and observed under a Nikon Labophot–2 microscope.

Material for electron microscopy

The observations used samples collected at the same time as for light microscopy. Samples were fixed overnight at 4˚C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.0 and postfixed for 3 h with 2% OsO_4 in 0.1 M cacodylate buffer. The material was then rinsed in 0.1 M cacodylate buffer (1.5 h) and twice (10 min) in distilled water. Samples were embedded in 1.5% (w/v) agar to facilitate handling and embedded in Spurr resin. Ultrathin sections stained with uranyl acetate and lead citrate were examined in a JEOL JEM–100Sx electron microscope.

RESULTS

OBSERVATIONS BY LIGHT MICROSCOPY

Just after isolation (day 0), the suspension culture contained mainly living microspores at the late uninuclear stage and pollen at the early bicellular stage. There were also many dead microspores.

After 5 to 7 days of culture, most microspores did not express any sign of development or else followed the gametophytic pathway of development. A small number of microspores significantly increased in size, but their enlargement was not a marker of microspore development. Cellularization, the number of nuclei and the general appearance were highly variable among the enlarged microspores as well as those that did not increase in size. There were microspores containing only a few nuclei (sometimes the nuclei were at one pole of the grain) as well as microspores with numerous nuclei (Fig. 1).

DAPI staining revealed differences between multinuclear/multicellular structures. In some structures, all nuclei were of the same size. Such structures were designated as one-domain. In other structures, two types of nuclei were present: large and small. Each group of nuclei of the same type formed a separate domain. The distances between large nuclei were bigger than between small nuclei; this may reflect differences in the dimensions of cells in two domains (Fig. 1).

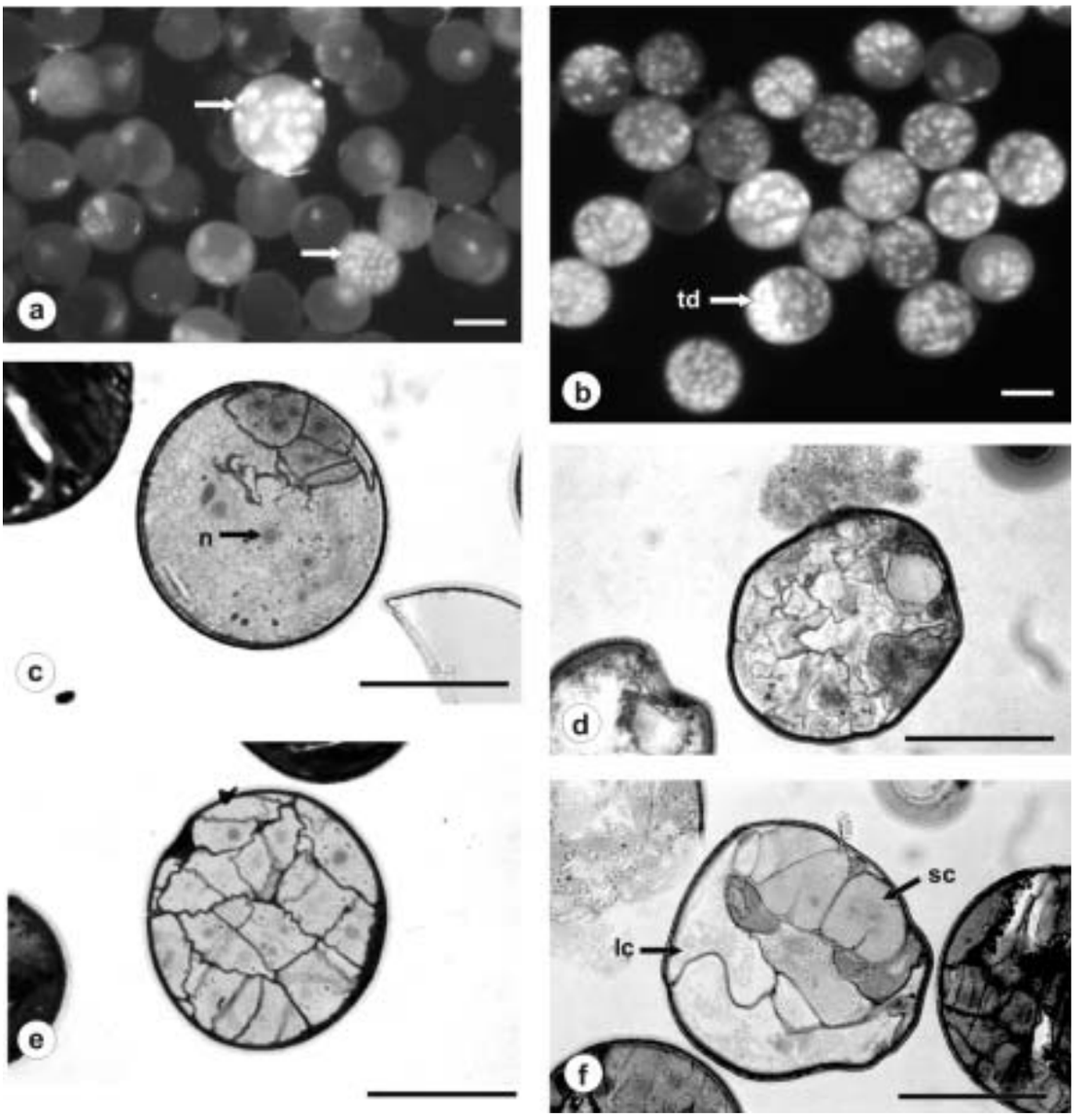


Fig. 1. Structures present in culture 5 to 7 days after microspore isolation. (**a**) General view of the culture, with most microspores arrested in development, two structures (arrows) containing numerous nuclei which are distinctly larger in one of them, (**b**) Several multinuclear structures differing in appearance, some (td) containing two different-looking domains, (**c**) Structure partially cellularized, the largest part of the grain containing free nuclei (n), (**d**) Structure containing highly waved cell walls, with cells difficult to distinguish, (**e**) Multicellular structure with slightly waved cell walls, the cells being clearly formed, (**f**) Two-domain structure, one domain containing small cells (sc), the other domain containing larger cells (lc). (a,b) DAPI staining, (c–f) semithin sections. Bars = 50 µm.

Analysis of semithin sections revealed the detailed cytological characteristics of the examined structures. The degree of differentiation markedly increased during culturing. In these structures, the cells occupied the whole space of the grain or only part of its volume (Fig. 1c,e). Cellular characteristics were very apparent. In most cases it was possible to identify whether there were one-domain or two-domain structures (Fig. 1e,f). In some cases, cellularization was more advanced at one pole than in the other part of the structure. Sometimes the process of wall formation was not completed and only part of the cytoplasm was partitioned into

cells, while the rest of the cytoplasm was a coenocyte (Fig. 1c). Free nuclei were located around the central vacuole or were randomly distributed in the cytoplasm.

Within structures enclosed in exine, characteristic wave-walled cells were frequently present. The degree of cellularization inside such grains varied. Sometimes the grain was completely divided into cells (Fig. 1f), and in other cases the wave-walled cells occupied only part of the grain (Fig. 1d).

After two weeks of culture, the heterogeneity of the culture had clearly increased. Non-induced microspores and multinuclear/multicellular structures enclosed in the exine were still present, but developing androgenic structures were also observed. They had ruptured the pollen wall and formed large multicellular masses (Fig. 2). These structures varied in their appearance and stage of development. Thus, the microspore derivatives reached a new stage of differentiation after release from the pollen wall. On the basis of cell size and the shape and general appearance of whole structures, three main morphological types were distinguished: (i) one-domain, (ii) two-domain, and (iii) callus-like structures.

As in the case of the structures enclosed in exine, one-domain structures contained only one type of cells (large or small). Usually the structures with small cells were compact and dark because the cells were tightly packed and had dense cytoplasm (Fig. 2c,e). In contrast, the structures with large cells were light in appearance due to a high degree of cell vacuolization (Fig. 2c,e).

In two-domain structures, each domain contained different types of cells. The shape and size of the domains varied conspicuously (Fig. 2a,b,d,e).

Callus-like structures formed clusters of rounded or elongated, loosely connected, highly vacuolated cells. The cells at the periphery of the cluster were probably released into the medium. In such structures, large intercellular spaces were present (Fig. 2e,f).

The structures described above represent the main types of androgenic structures observed in culture. There was also a variety of structures difficult to classify.

In one-month-old cultures, androgenic structures were large enough to be seen macroscopically (Fig. 3a). Some structures were many-fold larger than others and were of diverse shapes. Nevertheless, two-domain structures were the most frequent type observed. We did not observe the typical structures of a zygotic monocot embryo.

In macroscopic two-domain structures, the cell characteristics were connected with their localization in the domain. Analysis of semithin sections revealed that, as in the previous stages, large cells occurred in one domain while small cells with dark cytoplasm were present in another domain (Fig. 3c,d). At that stage, meristematic regions were observed within both domains. Callusing of some structures was observed (Fig. 3b,d).

ELECTRON MICROSCOPE OBSERVATIONS

Structures collected at days 7 and 12 of culture were analyzed ultrastructurally. The large cells are generally distinguishable as cells with light cytoplasm, and the small cells as cells with dark cytoplasm.

Ultrastructure of wave-walled cells

This type of cell was observed only in 7-day-old structures. The most striking feature of such cells was the presence of highly waved walls which sometimes formed a very complicated labyrinth inside the grain. The nuclei were not always completely separated from each other, so multinuclear compartments occurred. When the process of wall formation had been completed, the resulting cells were diverse in shape and size. Plastids with starch grains and lipid droplets were present in the cytoplasm (Fig. 4a).

Ultrastructure of small cells

The cytoplasm in small cells contained many tiny, irregularly shaped vacuoles; the level of vacuolization was variable. In some cells, many vesicles and lipid droplets were present in the cytoplasm. The plastids usually were not built of many thylakoids, but contained a few small starch grains. The cytoplasm was abundant in mitochondria and ribosomes. The mitochondria were round, sometimes elongated, and contained a variable number of tubular cristae. Except for ribosomes attached to the ER, many of them remained free; this may explain the dark appearance of the cytoplasm (Fig. 4b,c,d).

Ultrastructure of large cells

In large cells, the vacuoles usually occupied the main part of the cell volume, especially in 12-day-old structures. The cytoplasm was light, granulated, and rich in ER. Frequently, the ER formed long strands along the cell walls. The walls of some cells in direct contact with nutrient medium had folded surfaces. The mitochondria and plastids were similar to those observed in small cells, but the plastids usually had fewer starch grains (Fig. 4b,c)

Ultrastructure of cells from callus-like structures

Callus-like structures were observed only in cultures two weeks old and older. The characteristic feature of the cells forming such clusters was their round shape. The cells were sometimes multinuclear. The ground cytoplasm was electron-light and contained many vacuoles of diverse shape. Most mitochondria were round and cristae-rich. The ER was well developed and variable. In some cells the ER formed long strands, and in others it appeared as a large number of small cisternae.

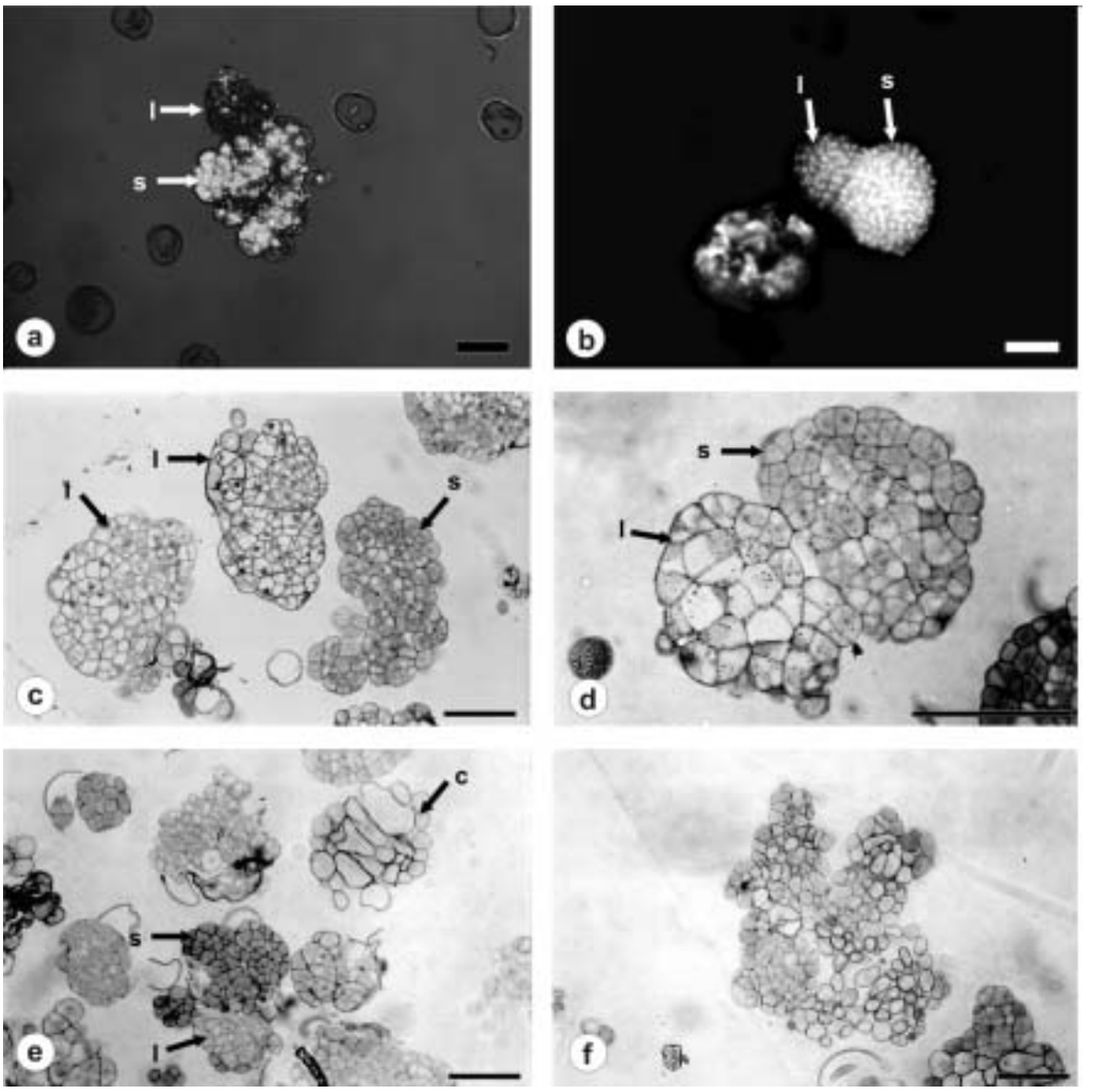


Fig. 2. Structures present in culture after 12 days of culturing. (**a**) Two-domain structure, the domains differing in nuclear size and density, and the cells seeming loosely connected, (**b**) Two-domain structure, more compact than the previous one, the domains differing mainly in nuclear density, (**c**) Three large multicellular structures, two of them containing large, vacuolated cells (l), and one containing small cells (s) with tiny vacuoles, (**d**) Two-domain structure, with one domain having large, vacuolated cells (l) and the other containing small cells (s) with tiny vacuoles, (**e**) Different types of multicellular structures, with one structure having two domains visible, one having small (s) and another having large (l) cells, and a callus-like structure (c), (**f**) Callus-like structure, the cells being rounded and loosely connected, with many intercellular spaces in the structure. (a,b) DAPI staining, (c–f) semithin sections. Bars = 100 μm.

Golgi complexes were frequent and contained many vesicles. An interesting feature of some cells in callus-like clusters was the presence of many vesicles joined to the outer cell membrane. There were a few lipid droplets and poorly developed plastids containing a few starch grains (Fig. 3e).

DISCUSSION

In higher plants, microspores undergo an ordered sequence of mitotic cell divisions which lead to the formation of pollen grains consisting of a vegetative cell and a generative cell, or two sperm cells, committed to specialized functions.

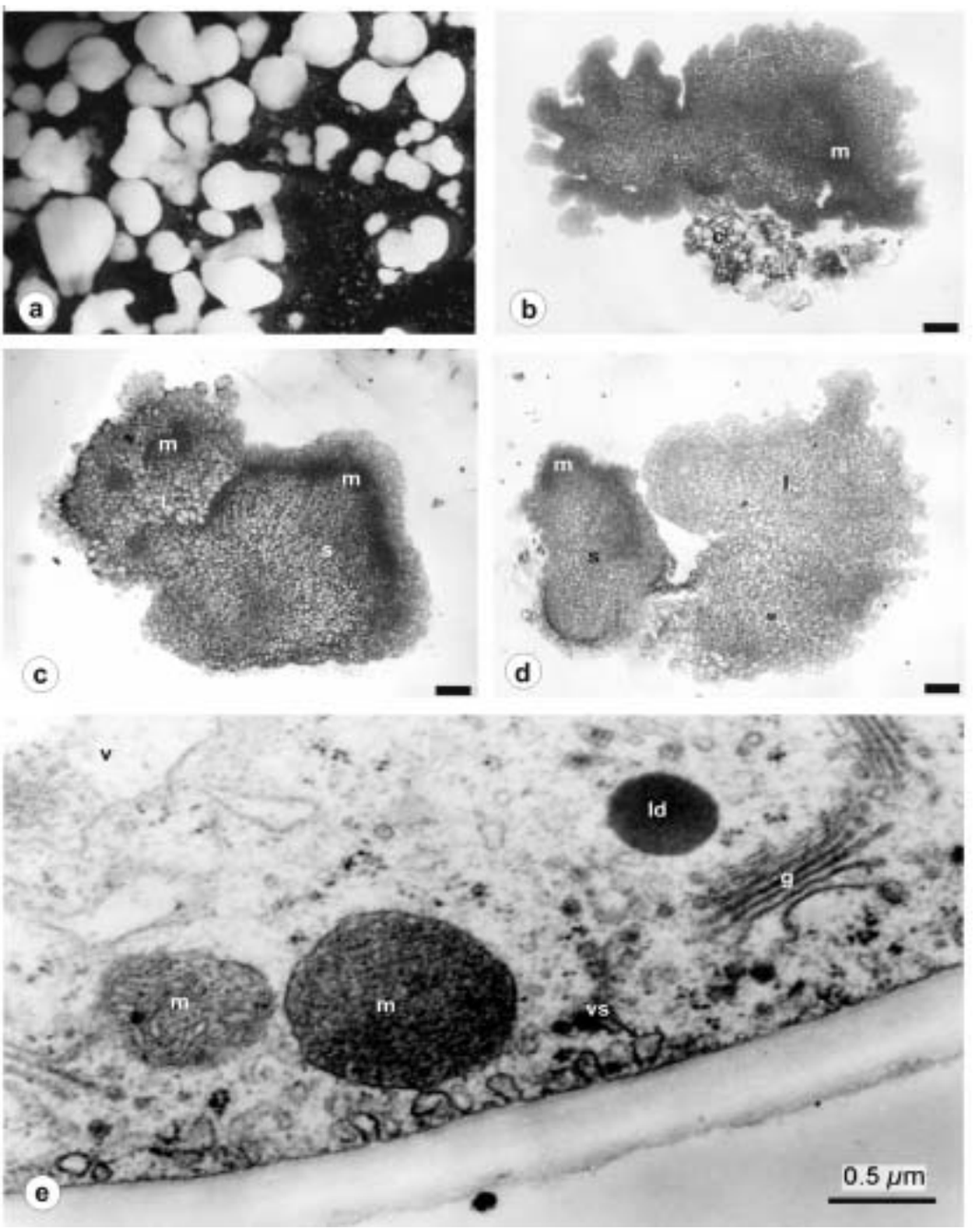
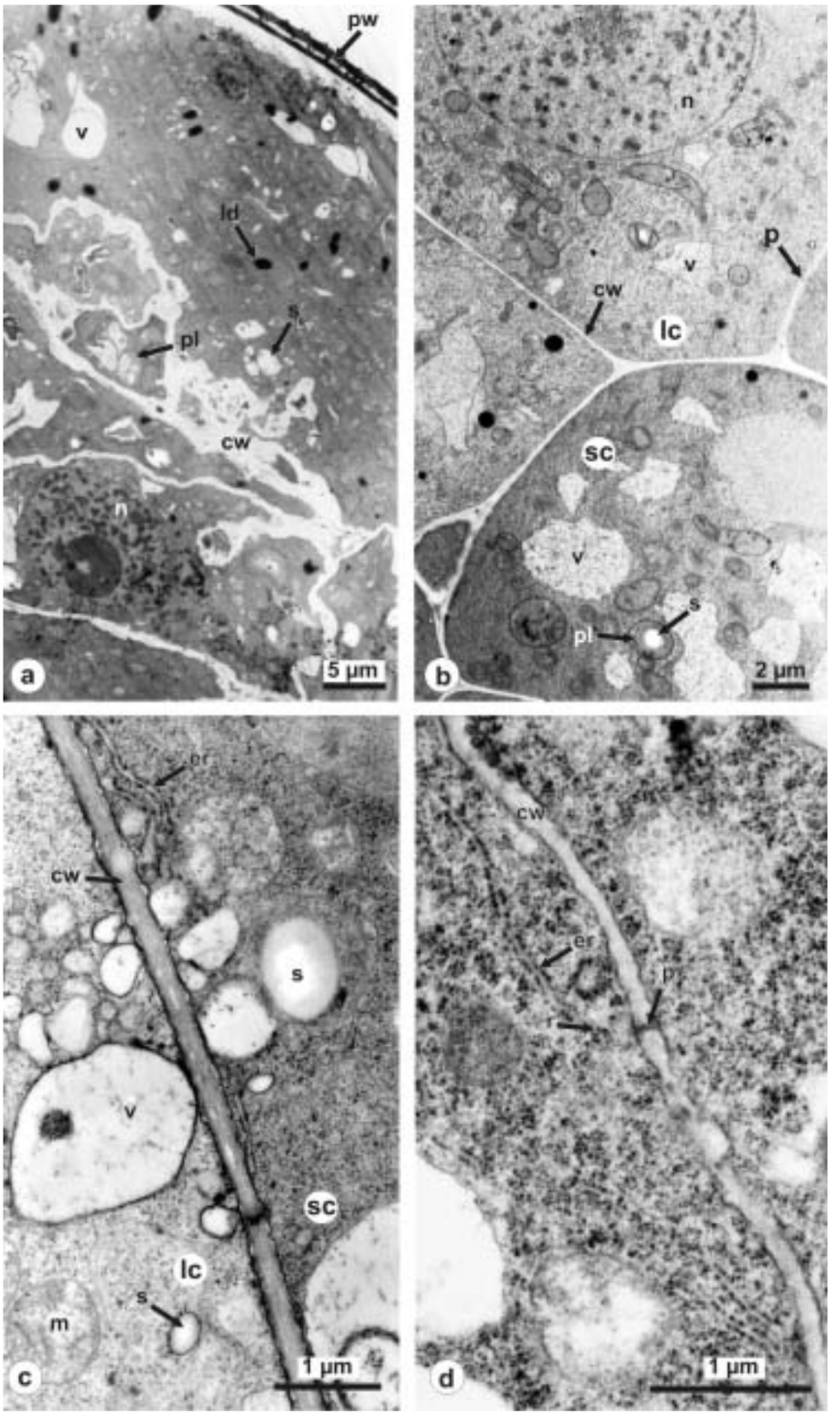


Fig. 3. Macroscopic structures present in microspore culture after 30 days of cultivation. (**a**) General view of the culture, with structures of different size and shape visible, (**b–d**) Different types of macroscopic structures. l – large-celled domain; m – meristematic region; s – small-celled domain. Bars = 100 μm, (**e**) Cell ultrastructure of 12-day-old microspore-derived callus-like cluster, with mitochondria rich in cristae, lipid droplets, vesicles attached to the peripheral cell wall, and many Golgi complexes. g – Golgi complexes; ld – lipid droplet; m - mitochondrion; v – vacuole; vs – vesicle.

Fig. 4. Cell ultrastructure of 7- and 12-day-old microspore-derived structures. (a,d) After 7 days of culture, (b,c) After 12 days of culture. (**a**) Fragment of wave-walled cell, the wall forming a complicated network, (**b–c**) Fragments of small (sc) and large (lc) cells, with light cytoplasm in large cells, darker cytoplasm in small cells, the organelles being similar, (**d**) Fragments of cells with dark cytoplasm rich in ribosomes. cw – cell wall; er – endoplasmic reticulum; ld – lipid droplet; m – mitochondrion; n – nucleus; p – plasmodesmata; pl – plastid; pw – pollen wall; r – ribosome; s – starch grain; v – vacuole.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The formation of embryos from microspores or pollen represents a fundamental switch in this development.

Numerous studies have shown that different cellular events accompany the transition from the gametophytic to the sporophytic pathway: alterations in the cell cycle, changes in the symmetry of cell division and reorganization of the cytoskeleton, vacuolization of the cytoplasm, a decrease in the ribosomal population, and the dedifferentiation of organelles (for review see: Rodríguez-Garcia et al., 2000; Smýkal, 2000; Touraev et al. 2000).

The characteristic feature of maize microspore culture observed in this study was its high heterogeneity. This variability occurred during the whole period of culture and was observed at the levels of light and electron microscopy. In some of the young androgenic structures, two domains of different size and cellular characteristics were easily recognizable. The general appearance of these structures, at least at 1–2 weeks of culture, seems similar to that of young zygotic embryos cultured in vitro. One domain, which is compact and dark, mimics the embryo proper; the second domain, built of larger, lighter cells, displays similarities to a suspensor (compare: Leduc et al. 1996).

These results are similar to previous reports of microspore and anther culture in other species. Raghavan (1976) first described the existence of two regions with various cellular characteristics in young microspore embryos of *Hyoscyamus niger*, suggesting that one region was homologous to the suspensor in zygotic embryogenesis. Later, the occurrence of different cell types in the early stages of androgenesis was reported in barley and wheat (Sunderland et al., 1979; Sunderland and Huang, 1985; Huang, 1986; Ramírez et al., 2001).

More recently, Testillano et al. (2002) published data concerning two-domain structures appearing in maize microspore culture. These authors distinguished four morphological types of structures containing two domains. Ultrastructural, cytochemical and immunocytochemical analyses of structures 5 to 7 days old revealed differences in the cellular organization of the domains. According to these authors, two-domain structures represent different stages in microspore-derived embryo development. The so-called "large domain" that occupied most of the grain shares characteristics with the endosperm in zygotic embryogenesis in the way of development, cellular organization, and features at different stages. These similarities include the coenocytic stage of domain, the presence of a large central vacuole, "free growing" cell walls, the appearance of callose in cell walls, relatively high tubulin capacity, and the presence of starch. On the other hand, the "small domain" was built of cells with dense cytoplasm with scarce vacuoles, a large nucleus, and not very thin cell walls with plasmodesmata. These cellular features mimic characteristics of embryo proper cells at the first stages of zygotic embryogenesis.

Similarity to the endosperm-like functions of young microspore-derived maize embryos was reported earlier by Magnard et al. (2000). The *ZmAE1* and *ZmAE3* genes expressed in the embryo-surrounding region (ESR) of the endosperm during zygotic embryogenesis were found to be expressed in androgenic structures as well. These results were later confirmed by Testillano et al. (2002) and Sevilla-Lecoq et al. (2003).

A characteristic feature of maize microspore culture is its high degree of variability, which could be observed throughout the period of culture. Different types of structures or their domains can be compared to the embryo proper, suspensor or endosperm. The source of such heterogeneity might be connected with differences in gene activation during the inductive stage of androgenesis and with the genome of the microspores themselves. From this point of view, microspore derivatives might be treated not only as embryos of androgenic origin but also as a population of structures with a wide spectrum of cytological, histological, developmental and genetic features. Future studies should analyze gene activity pattern(s) in different types of microspores and microspore-derived structures.

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