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# Embryological and cytological features of *Gagea bohemica* (Liliaceae)

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**Abstract:** The present study describes the developmental features of the embryo sac and ovular structures, particularly the obturator, during development in *Gagea bohemica* (Zauschn.) Schult. & Schult. f. The nucellar epidermis is poorly developed and composed of 1-2 layers of cuboidal cells. The tissue at the chalazal end of the nucellus differentiates into a hypostase. The micropyle is formed by the inner integument and composed of 4-5 cell layers that include starch grains. The functional megaspore results in an 8-nucleated embryo sac and conforms to a tetrasporic, *Fritillaria* L. type. Cytoplasmic nucleoloids consisting of protein and RNA are obvious during megasporogenesis. The obturator attracts attention during embryo sac development. Cytochemical tests indicated that the cells of the obturator present a strong reaction in terms of insoluble polysaccharide, lipid, and protein. Obturator cells are coated by a smooth and thick surface layer that starts to accumulate partially and then merges. Ultrastructural studies reveal that obturator cells are rich in rough endoplasmic reticulum, polysomes, plastids with osmiophilic inclusions, dictyosomes with large vesicles, mitochondria, and osmiophilic secretory granules. After fertilisation, the vacuolisation in obturator cells increases by fusing small vacuoles to form larger ones. Some of the small vacuoles contain electron dense deposits. Afterwards, the obturator cells shrink and disappear.

**Key words:** *Gagea bohemica*, embryo sac, nucleoloid, obturator

## Introduction

The genus *Gagea* Salisb. of the family Liliaceae comprises between 70 and 250 species, depending on the taxonomic conceptions of the authors (Mabberley, 1997; Tamura, 1998; Levichev, 1999), and appears in Europe, Asia, and North Africa (Caparelli et al., 2006). The high variation of morphological characters and the superficial similarity of most of the species have made taxonomic division of the genus uncertain and problematic.

The genus *Gagea* has been the object of karyological (Peruzzi, 2003; Peruzzi & Aquaro, 2005) and palynological (Zarrei & Zarre, 2005) investigations. Although there have been satisfactory embryological studies done on *Gagea lutea* (L.) Ker. Gawler (Bohdanowicz & Lewandowska, 1999; Bohdanowicz et al., 2005) and on *G. fascicularis* Salisb. (Joshi, 1940), little is known about the embryology, cytochemistry, and ultrastructure of other *Gagea* species, including *Gagea bohemica* (Zauschn.) Schult. & Schult.f., *G. bohemica*, also known as the Early Star-of-Bethlehem or Radnor Lily, is a yellow flowering plant that grows mainly on dry grassland. Caparelli et al. (2006) produced

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a comparative analysis of embryo sac development in 3 closely related *Gagea* species (*G. bohemica*, *G. chrysantha* (Jan) Schult. & Schult.f., and *G. granatellii* (Parl.) Parl.) with some consideration given to their reproductive strategies. However, those researchers failed to provide any mention of the ovular structures or the cytochemical and ultrastructural features of these reproductive cells. Peterson et al. (2010) studied the morphological and molecular aspects of *Gagea bohemica*, which is thought to consist of 2 extreme forms (saxatilis and bohemica).

The reproductive and embryological knowledge of plants provides useful data for the fields of cell biology, reproductive ecology, and taxonomy, as well as being invaluable for purposes related to seed production and cross-breeding. Although inflorescence, flower colour, style shape and structure, filament structure, megasporogenesis, and megagametogenesis have received the most attention for characters of taxonomic value in the angiosperms, other ovular features such as the number of integuments, position of megaspore mother cell in the nucellus, presence or absence of endothelium, aril, obturator, and nucellar cap are essential for their ability to reveal taxonomic distinction (Stuessy, 2009; Tekşen & Aytaç, 2011). Recently, the use of nuclear and plastid DNA markers has been put forward as a way of presenting phylogenetic relationships (İkinci, 2011).

In light of the above mentioned criteria, we aimed to compose a detailed report on the cytochemical and ultrastructural features of embryo sac and ovular structures, particularly the obturator, in the course of the development of *Gagea bohemica*. Information on the development of the female reproductive structures in *Gagea bohemica* will help advance our understanding of its reproductive behaviour and will thus contribute to attempts to solve taxonomic problems in *Gagea* species.

## Material and methods

Flower buds of *Gagea bohemica* (Liliaceae) growing in natural habitats in the vicinity of Başıbüyük-İstanbul (Turkey) were collected in March and April. Flower buds were fixed in acetic acid:alcohol (1:3, v/v) for 24 h at room temperature. After dehydration in a graded series of ethanol, the material was embedded in paraffin. Sections (8-10 μm) were cut using a Leica RM2125RT microtome and stained with Delafield's hematoxylin.

For ultrastructural studies, flower buds were fixed in Karnowsky fixative (5% glutaraldehyde and 4% paraformaldehyde) in 0.1 M cacodylate buffer (pH 7.4) for 24 h at 4 °C and post-fixed in 1% osmium tetroxide in the same buffer for 1.5 h at room temperature. The samples were dehydrated in an ethanol series, and embedded in epoxy resin using propylene oxide. Ultrathin sections (~70 nm) were cut using a Leica Ultracut R, contrasted with uranyl acetate and lead citrate, and examined with a JEOL JEM 1011 transmission electron microscope (TEM).

In order to perform cytochemical observations, the osmication step was omitted from the fixation. Semi-thin sections (1 μm) were stained with 1% periodic acid-Schiff (PAS) (Feder & O'Brien, 1968) for insoluble polysaccharides, 0.2% Coomassie Brilliant Blue (Fisher, 1968) for proteins, 1% Sudan Black B for lipids (Pearse, 1961), and 0.025% Azure B (Jensen, 1962) for RNA. The sections were photographed with ProgRes CapturePro 2.6 software, assisted by a Jenoptik 122CU colour camera and an Olympus BX-51 microscope.

## Results

In *Gagea bohemica* the ovary is trilocular and ovules are located in each loculus. The mature ovule is anatropous, bitegmic, and tenuinucellate. The inner integument initiates first and then the outer integument develops into a small protuberance. Integuments usually consist of 2 layers of cells. The micropyle is formed by the inner integument alone because the outer integument remains short (Figure 1). The micropylar part of the inner integument is composed of 4-5 cell layers that include numerous starch grains at the stage of mature embryo sac (ES) (Figure 1). The outer integument and funiculus also contain numerous starch grains (Figure 1).

The nucellar epidermis is poorly developed and composed of 1-2 layers of cuboidal cells. The tissue at the chalazal end of the nucellus differentiates into a hypostase as the ovule develops. The first hypostase cells are visible at the 2-nucleate ES stage. The number of cells increases in further stages and becomes more

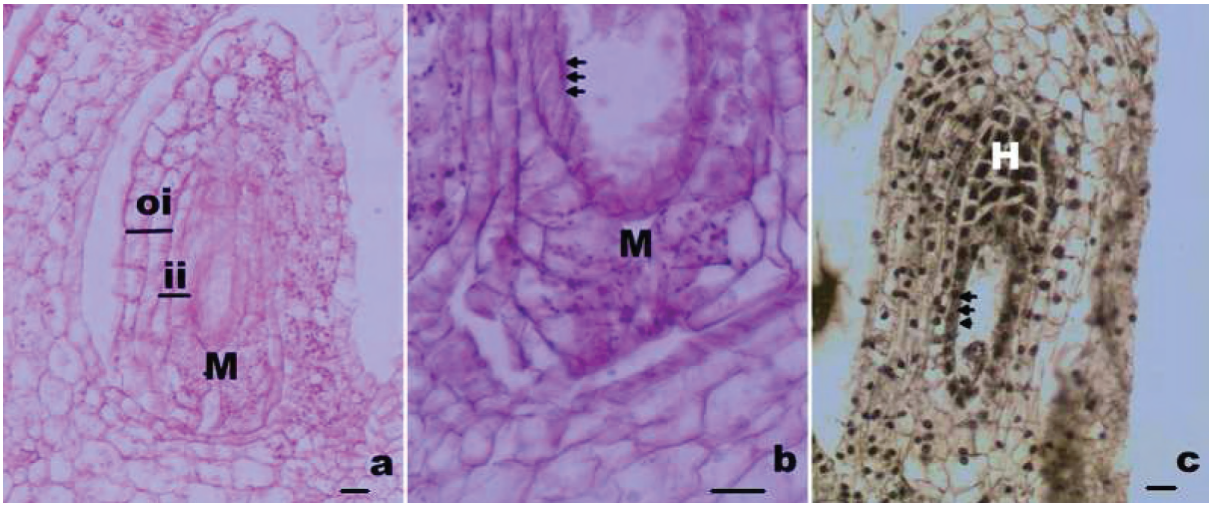


Figure 1. Ovular structures of *Gagea bohemica*. **a** - positions of micropyle, inner and outer integument stained with PAS; **b** - PAS positive reaction in endothelial cells (arrows), numerous starch grains in micropylar part; **c** - thick walled hypostase cells stained with hematoxylin. Note the endothelium with cuboidal cells (arrows). H: hypostase, ii: inner integument, M: micropyle, oi: outer integument. Scale bars = 10 μm.

prominent in the 8-nucleate ES. The hypostase cells are thick walled, each containing dense cytoplasm and a prominent nucleus with a few nucleoli (Figure 1).

The inner parietal layers of the integument differentiate into a thin layer of endothelium with cuboidal cells and dense cytoplasm. Single layered endothelial cells demonstrated a strong PAS positive reaction (Figure 1).

The ovular primordium consists of a compact homogeneous mass of parenchyma in which a single sub-epidermal cell differentiates as the female archesporium. The archesporial cell enlarges considerably and functions directly as the megaspore mother cell (MMC), which undergoes regular meiosis to form a dyad and then a linear tetrad (Figure 2). As ovule growth progresses, the ovule becomes anatropous since integument growth is more rapid on one side than on the other. The 4 nuclei are arranged in 1 row or, rarely, in a zigzag fashion, depending on the breadth of the ES (Figure 2). Subsequently, 3 of the 4 nuclei migrate to the chalazal end; nuclear arrangement afterward is 3 + 1 (Figure 2). Triple fusion in the chalazal end leads to the development of a 3n nucleus. Afterwards, mitotic divisions in the functional megaspore result in an 8-nucleated ES that conforms to the *Fritillaria* type (Figure 2). The antipodals in the chalazal end degenerate earlier and 2 antipodals are therefore monitored in the ES in most cases (Figure 2). Polar nuclei lie below the antipodals and they soon fuse to form the secondary nucleus (Figure 2). The secondary nucleus is close to the antipodals and is 4n. In a mature ES, the egg apparatus consists of 1 egg cell and 2 synergids. In the course of fertilisation, however, 1 of these synergids degenerates (Figure 2).

Light microscopic studies reveal cytoplasmic nucleoloids consisting of protein and RNA. These results were obtained with Coomassie Brilliant Blue (Figure 3) and Azure B (Figure 3) during megasporogenesis.

The obturator, which is formed at the base of the funiculus and at the tip of the carpel margin, attracts attention during ES development. The initial indication of obturator development is concurrent with prophase I of megasporocyte meiosis. As development progresses, the obturator forms a pad of tissue that lies vertically from the locule base to slightly above the apical ovule. The obturator consists of thick-walled, columnar, and glandular cells. Before fertilisation, the mature obturator cells with dense cytoplasm contain a spherical and massive nucleus and small vacuoles (Figure 4). After fertilisation (zygote in the embryo sac), vacuolisation progresses

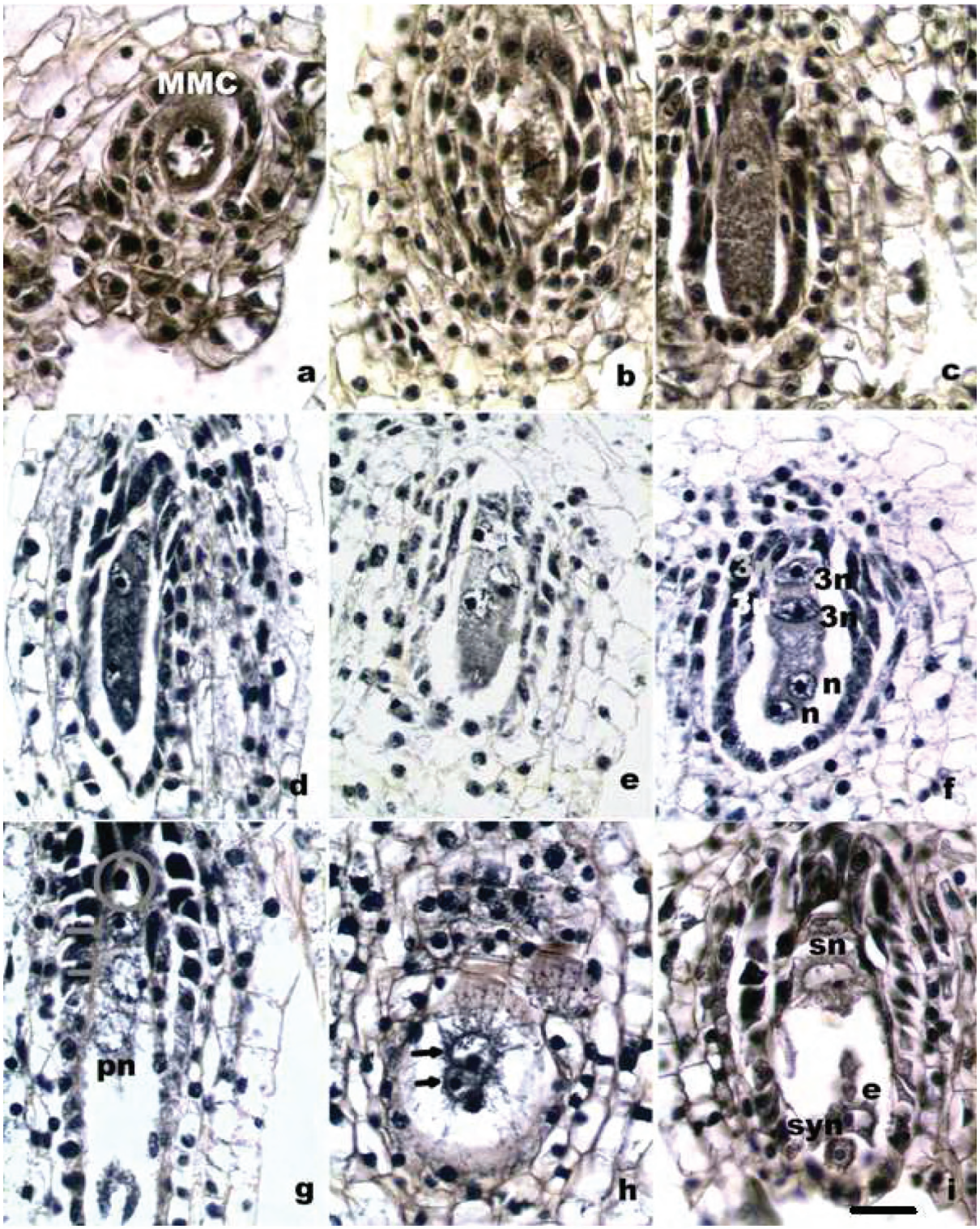


Figure 2. Megasporogenesis and development of the embryo sac in *Gagea bohemica*. **a** - megaspore mother cell (MMC); **b** - metaphase I; **c** - binucleate embryo sac; **d** - Linear megaspore tetrad; **e** - 3 + 1 arrangement; **f** - four nucleate embryo sac (*Fritillaria* type); **g** - degenerating chalazal antipodal (in circle), 2 active antipodals (arrows) and polar nucleus (pn); **h** - fusion of polar nuclei (arrows); **i** - secondary nucleus (sn). syn: synergid, e: egg cell. Scale bar = 10 μm.

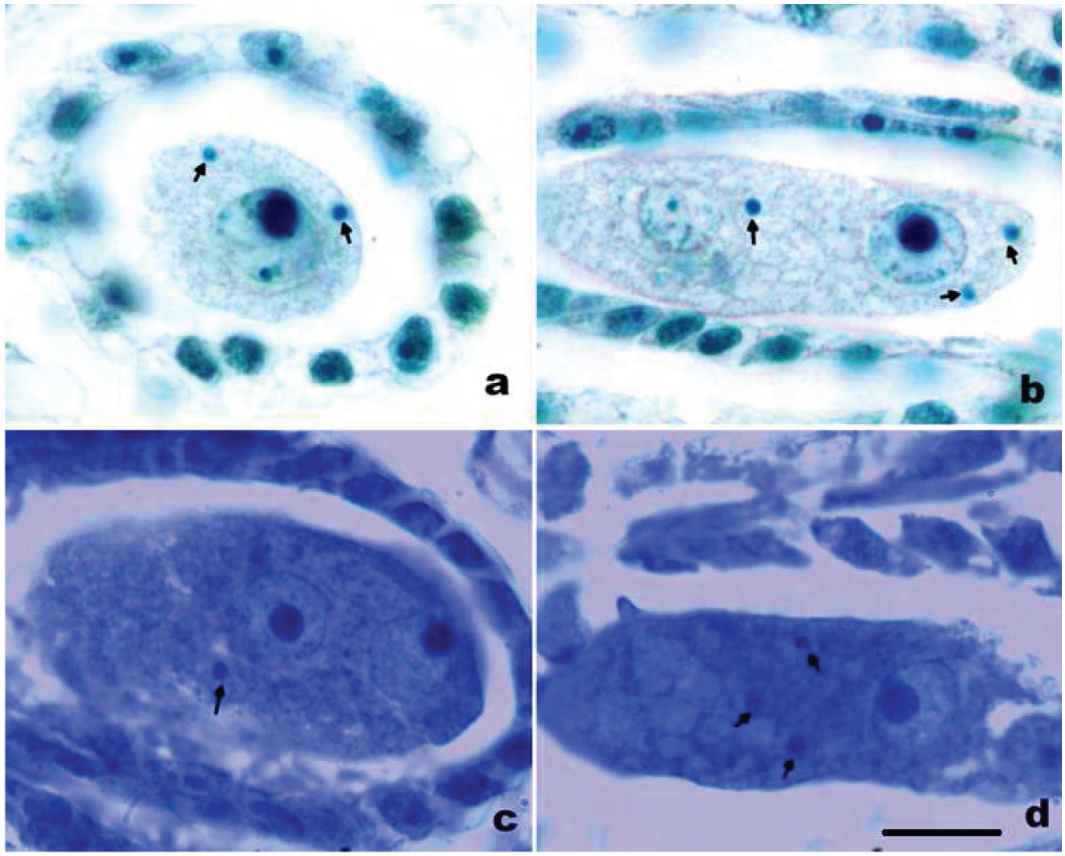


Figure 3. Nucleoloids (arrows) in embryo sac stained with Coomassie Brilliant Blue (**a, b**) and Azure B (**c, d**). Scale bar = 10 μm.

through the fusion of small vacuoles in the cells of the obturator. According to the vacuolisation the cytoplasm is displaced to the periphery of the cell and the large vacuole appears in the distal end of the cell (Figure 4). Cytochemical tests indicated that the cells present a strong reaction with regard to insoluble polysaccharide, lipid, and protein, as determined through the use of PAS (Figure 4), Sudan Black B (Figure 4), and Coomassie Brilliant Blue (Figure 4), respectively. The secretory substances form a smooth, thick layer on the cell surface. As evidenced cytochemically, the surface layer at the locular side consisted of dense insoluble polysaccharide and lipid (Figure 4). After fertilisation, the surface layer becomes thicker and presents invaginations. Although the lipid and protein contents of the surface layer present slight alteration (Figure 4), it is obvious that insoluble polysaccharides increase and invaginations are distinct (Figure 4). Afterwards, the obturator cells shrink and disappear.

The detailed studies of transmission electron microscopy confirm that cytoplasm in the obturator cells is dense and contains numerous short strands of rough endoplasmic reticulum (RER), polysomes, plastids with dense stroma and osmiophilic inclusions, dictyosomes with large vesicles, mitochondria, and osmiophilic secretory granules of different sizes (Figure 5). Ultrastructural evidence revealed that the obturator is coated by a surface layer that is produced by its own cells. Osmiophilic secretory granules penetrate the cell wall, causing an increase in width (Figure 6). This coat starts to partially accumulate; by cell maturity, it merges and the obturator is covered with a smooth, thick layer (Figure 6). After fertilisation, the cell wall continues thickening and presents large invaginations (Figure 6).

After fertilisation, the vacuolisation increases by fusing small vacuoles to form larger ones in obturator

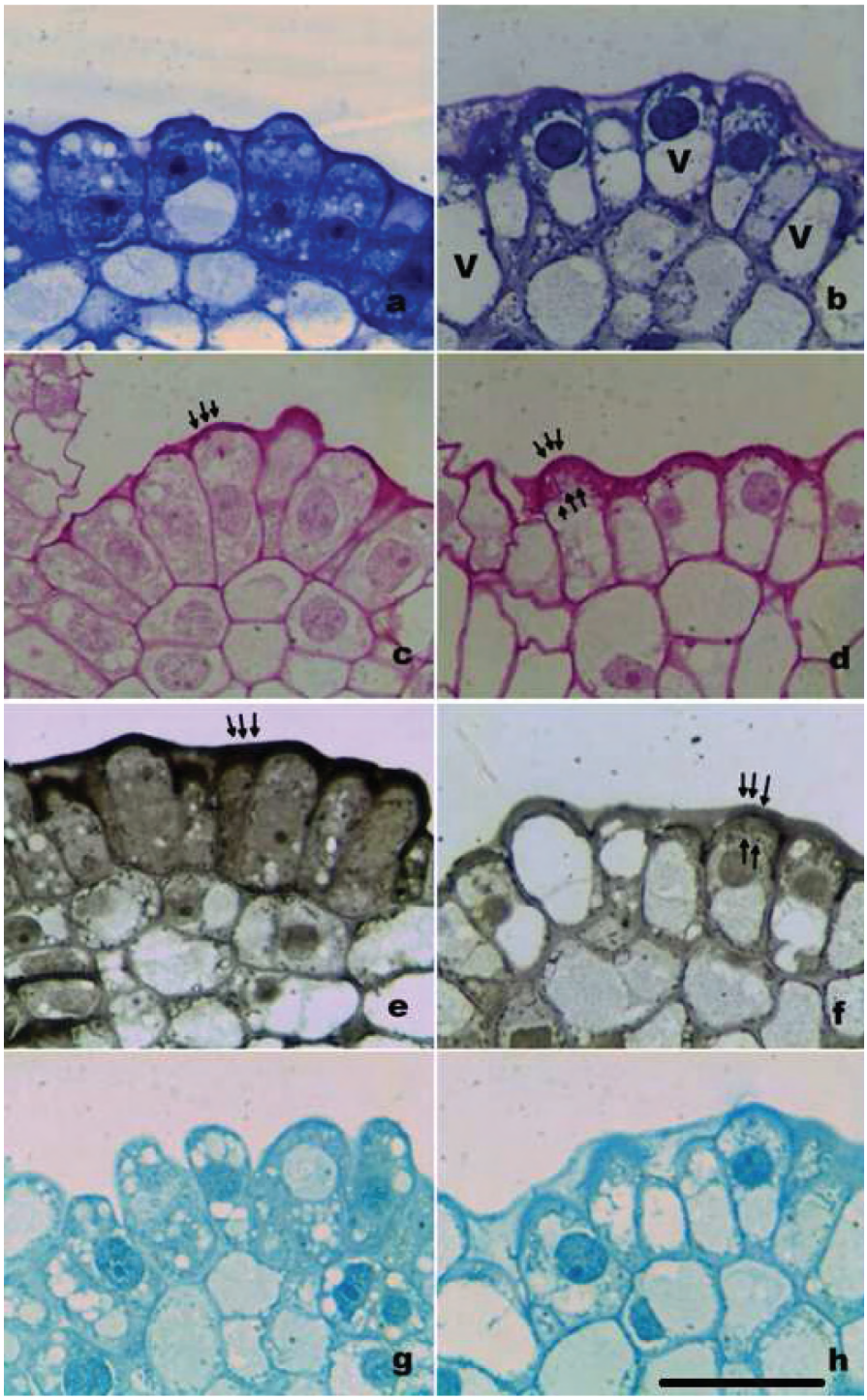


Figure 4. Development and cytochemistry of obturator cells. **a** - obturator cells before fertilisation stained with toluidine blue; **b** - large vacuoles (v) in the obturator cells after fertilisation stained with toluidine blue; **c** - obturator cells before fertilisation stained with PAS, arrows indicate PAS positive cell wall; **d** - obturator cells after fertilisation stained with PAS, arrows indicate PAS positive surface layer and invaginations; **e** - obturator cells before fertilisation stained with Sudan Black B, arrows indicate lipoidal surface layer; **f** - obturator cells after fertilisation stained with Sudan Black B, arrows indicate cell wall invaginations; **g** - obturator cells before fertilisation stained with Coomassie Brilliant Blue; **h** - obturator cells after fertilisation stained with Coomassie Brilliant Blue. Scale bar = 10 μm.

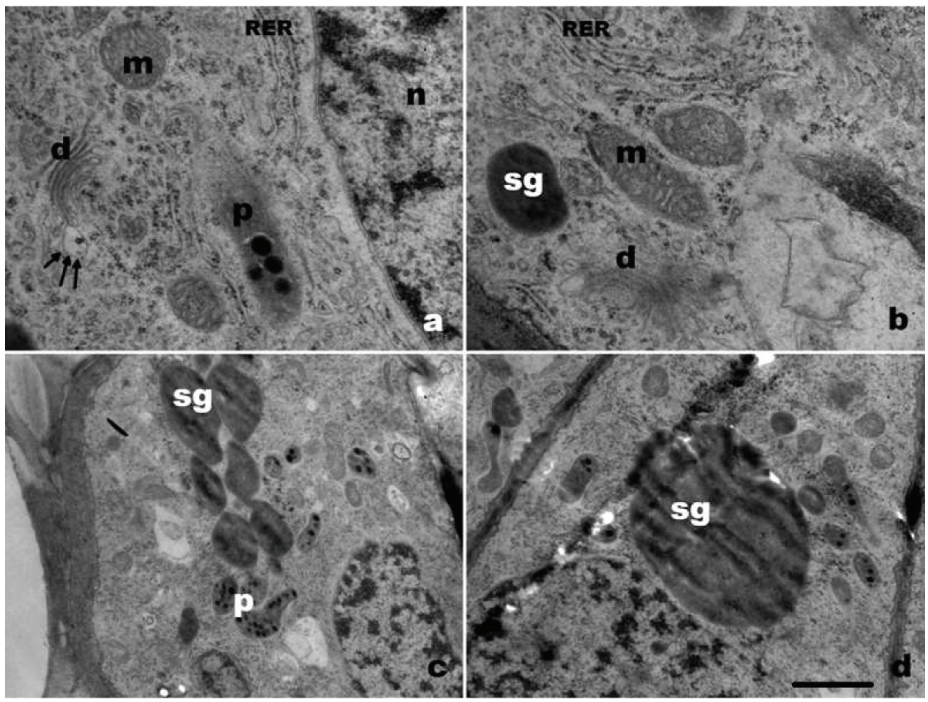


Figure 5. Electron micrographs of obturator cells before fertilisation (**a**-**d**). d: dictyosomes, p: plastids, n: nucleus, m: mitochondrion, RER: rough endoplasmic reticulum, sg: secretory granules. Scale bar = 0.5 μm.

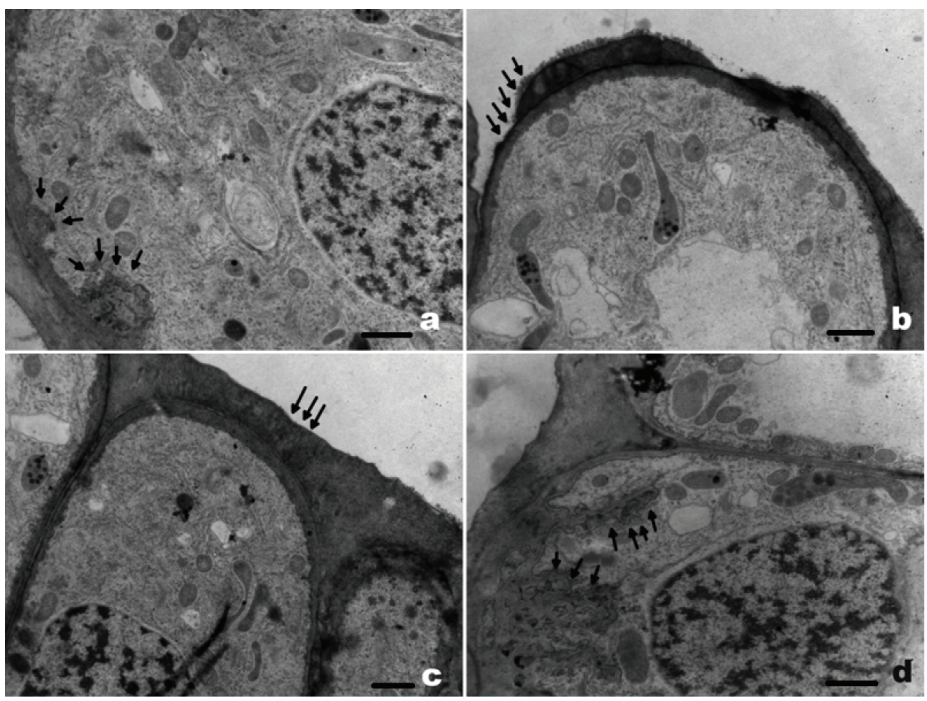


Figure 6. Formation of surface layer of obturator. **a** - penetration of secretory granules (arrows) to the cell wall; **b** - partially accumulating surface layer (arrows); **c** - thick surface layer (arrows); **d** - cell wall invaginations (arrows). Scale bars = 1 μm.

cells. Some of the small vacuoles contain electron-dense deposits encircled with membrane and that originated from cytoplasm (Figure 7). According to the large vacuoles, the cytoplasm and nucleus take part in the locular side of the cell (Figure 7). The plastids have also increased in size and contain starch grains (Figure 7).

## Discussion

We have presented light and electron microscopic descriptions of ovule development in *Gagea bohemica* with a consideration of cytochemical features.

The ovule of *G. bohemica* is anatropous, bitegmic, and tenuinucellate, with the micropyle formed by the inner integument, as is consistent with the family Liliaceae (Davis, 1966). According to our results, the archesporial cell functions directly as the MMC and ES conforms to the *Fritillaria* type. Caparelli et al. (2006) studied 3 species of *Gagea* and indicated that they follow the *Euphorbia dulcis* type of ES development. The researchers explained that the correct and priority name for the *Fritillaria* type (Martinoli, 1940; Battaglia, 1986), a widespread name still quoted by recent books of embryology (Batygina, 2002), is, in fact, *Euphorbia dulcis* type, which had been recognised, described, and characterised in 3 phases by Carano (1926), Chiarugi (1927), and Bambacioni (1928).

The obturator, which is described as a modification of ovarian tissue, mostly occurs in the family Liliaceae (Tilton & Horner, 1980; Zhou et al., 2004), as has been previously reported in *Ornithogalum caudatum* Jacq. (Tilton & Horner, 1980), *Allium peroninianum* Azn. (İsmailoğlu et al., 2010), *Scilla autumnalis* L. (Coşkun & Ünal, 2010), and *Ornithogalum sigmoideum* Freyn & Sint. (İsmailoğlu & Ünal, 2011). The obturator may originate from a variety of sources: the placenta (Bor & Bauman, 1974), the funiculus (Capus, 1978), the funiculus and placenta combined (Tilton & Horner, 1980), the integuments (Davis, 1966), or the arils (Rao, 1959). Obturators of different species have different morphological characters, such as a pad or swelling, hairs, filaments, or tufts (Tilton & Horner, 1980). In *Gagea bohemica*, the obturator originates from the base of the funiculus and forms a pad of tissue.

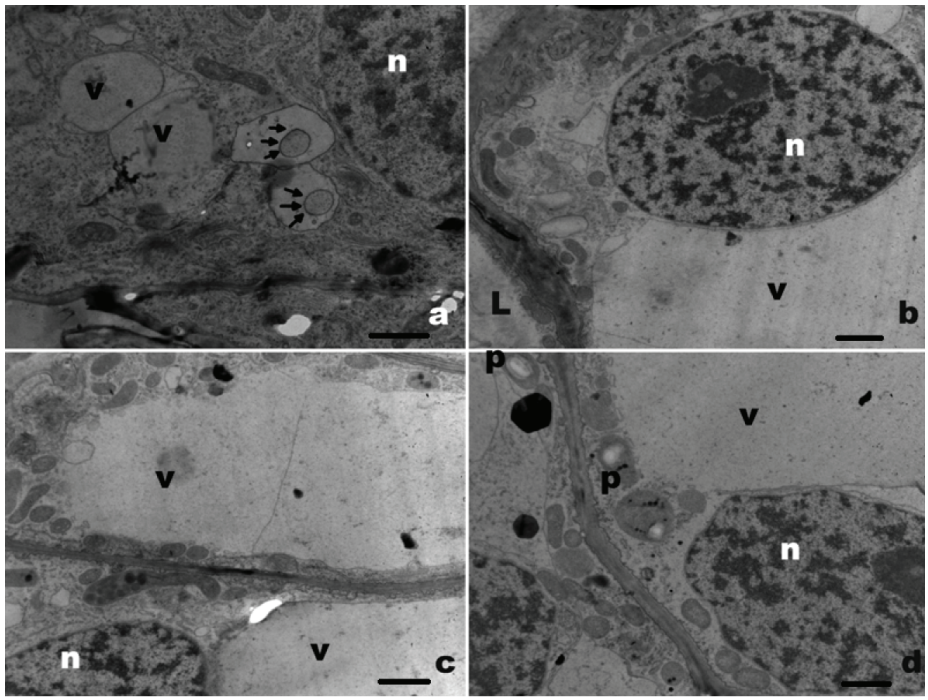


Figure 7. Vacuolisation after fertilisation in obturator cells. **a** - electron dense deposits (arrows) in vacuoles (v); **b, c** - according to the large vacuoles (v) the cytoplasm and nucleus (n) take part in the locular side of the cell; **d** - plastids (p) with starch grains. Scale bars = 1 μm.

It has been reported that the chief function of the obturator is to provide additional nutrients and further mechanical and chemical guidance to lead growing pollen tubes towards the micropyle (Tilton & Horner, 1980; van Rensburg & Robbertse, 1988). As evidenced by the cytochemical analysis carried out in this study, obturator cells in *G. bohemica* secrete substances containing insoluble polysaccharides and lipids and that cover the surface completely, playing a role in guiding pollen tubes to the ovule. In addition, the cells are rich for insoluble polysaccharides, protein, and lipids before fertilisation. After fertilisation, the content of organic compounds decreases due to vacuolisation.

While information is available on the morphology and function of the obturator in different species, information is lacking on the cytochemical and ultrastructural level. The role of the obturator in pollen tube growth in the ovary has been investigated often. Ultrastructural studies indicated that obturator cells were rich in RER, dictyosomes, ribosomes, plastids, and mitochondria in *G. bohemica*. To the best of our knowledge, no other report has been published including detailed descriptions of the cytochemistry and ultrastructure of the obturator cells.

After fertilisation, obturator cells increased in the vacuolisation of *G. bohemica*. Vacuolisation is conspicuous during programmed cell death during reproductive development, including that of the nucellus of *Ginkgo biloba* L. (Li et al., 2003), anther tapetum (Vardar & Ünal, 2012), and anther filament cells (Vardar & Ünal, 2011). Furthermore, pyknotic nucleus, DNA fragmentation, and condensation are of great importance in identifying plant-programmed cell death. Ultrastructural results revealed that no nuclear degeneration was observed during the development and degeneration of obturator cells. Although vacuolisation is conspicuous in the obturator cells of *G. bohemica*, because of the lack of DNA fragmentation and condensation, we concluded that the reason or type of the obturator degeneration is not programmed cell death.

Cytochemical results revealed obvious nucleoloids containing protein and RNA in the ovule of *G. bohemica*. The numerous small, nucleolus-like bodies present in the cytoplasm of reproductive cells of plants at meiotic stages have been called cytoplasmic nucleoloids (Dickinson & Heslop-Harrison, 1970). Kusanagi and Kawano (1975) reported nucleoloids to be present in the cytoplasm throughout meiosis, from diakinesis to telophase II in *Maianthemum dilatatum* A.Nelson & J.F.Macbr. Further examples are provided by the nucleoloids present in the cytoplasm during micro- and megasporogenesis in *Lilium* L. (Dickinson & Heslop-Harrison, 1970; Williams et al., 1973; Dickinson & Potter, 1978). Although nothing is known about the function of the nucleoloids, it has been suggested that since a clearly defined nucleolus is absent during these periods, these inclusions together may represent the nucleolar apparatus of the cell (Sato et al., 1991). However it is important to note that nucleoloids have not been observed in all species and thus cannot be considered a general feature of microsporogenesis or megasporogenesis. Cresti et al. (1992) regarded the nucleoloids as stored ribosomes. Further investigations confirmed the ribonucleoproteinic nature of the nucleoloids and the presence of rRNA transcripts (Alché et al., 1994).

In conclusion, the ES development of *G. bohemica* conforms to the *Fritillaria* type. Obturator cells came forward during ES development in response to structural development and organic (protein, insoluble polysaccharide, lipid) compound accumulation. Our data provide a new look at the sexual reproductive potential of *G. bohemica*, an example of the genus *Gagea*. The cytochemical and ultrastructural development of obturator cells will be the object of pollen tube growth investigations. Moreover, embryo sac features will provide useful characters for assessing relationships within this genus and family.

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