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Embryological and cytochemical features of *Scilla autumnalis* L.

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Abstract: Nucellar epidermal cells in the micropylar region of *Scilla autumnalis* L. differentiate into a nucellar cap. It secretes a PAS-positive exudate filling the micropyle. A group of cells at the chalazal end of the nucellus forms a hypostase. Close to the antipodals, the hypostase cells contain abundant starch grains, suggesting a function as a storage tissue. Another ovular structure is the obturator. It secretes an exudate that stains deeply with PAS reaction, and it takes a role in directing the growth of pollen tubes toward the micropyle. Cytochemical tests in the mature embryo sac indicate the presence of high amounts of proteins, polysaccharides, and lipids in the cytoplasm of the egg, synergids, antipodals, and central cell, appearing to be the site of intensive synthetic activity. The presence of a PAS-positive wall completely surrounding the egg cell and synergids is an unusual feature worthy of mention. Antipodals undergo polytenisation to reach ploidy levels of up to 128n. Another embryological feature of *S. autumnalis* is the development of an Onagrad type of embryo and a helobial type of endosperm.

Key words: *Scilla autumnalis*, embryo, endosperm, antipodals, cytochemistry

Scilla autumnalis L. türünün embriyolojik ve sitokimyasal özellikleri

Özet: *S. autumnalis* türünün mikropil tarafındaki nusellus epidermis hücreleri, nusellus şapkasını oluşturur. Nusellus epidermisi ve nusellus şapkası hücrelerinin hem çeperlerinin hem de sitoplazmalarının PAS pozitif reaksiyon verdiği ve sitoplazmalarının bol miktarda nişasta tanesi içerdiği saptandı. Gelişim sırasında, embriyo kesesinin kalaza tarafında hipostas adını alan doku farklılaşması gözlendi. Antipodlara yakın olan hipostas hücreleri bol miktarda nişasta taneleri içerir; bir depo görevi görürler. Tohum taslağında bulunan diğer bir yapı da funikulusun kaidesinde küçük bir çıkıntı halinde farklılaşan obturatordur. Oburator hücreleri bol nişasta içerirler, PAS ile kuvvetli reaksiyon verirler ve polen tüpünün mikropile doğru büyümesinde görevlidirler. Sitokimyasal testler, olgun embriyo kesesindeki yumurta, sinergitler, antipodlar ve merkez hücrenin sitoplazmalarında bol miktarda protein, polisakkarit ve lipit bulunduğunu gösterdi. Yumurta hücresi ve sinergit hücrelerinin etrafı kuvvetli PAS pozitif reaksiyonu veren tam çeperle çevrilidir. Antipotlar daimi yapılardır ve ritmik büyüme gösterirler. Antipot nukleuslarının hacimleri ploidi seviyesinin n-128n arasında değiştiğini gösterdi. *S. autumnalis*'in bir diğer embriyolojik özelliği de embriyo gelişiminin Onagrad tipte ve endospermasının helobial tipte olmasıdır.

Anahtar sözcükler: *Scilla autumnalis,* embriyo, endosperma, antipot, sitokimya

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Introduction

In the genus *Scilla* L., the development of the female gametophyte shows heterogeneity and mostly conforms to the Polygonum type, as in *S. autumnalis* L. (Battaglia, 1958b), *S. indica* (Govindappa & Sheriff, 1951; Sulbha, 1954), *S. obtusifolia* (Battaglia, 1958b), *S. pratensis* (Battaglia & Feeley, 1959), and *S. hyacinthina* (Sulbha, 1954). It follows the Endymion type of development in *S. hispanica* (=*Endymion hispanicus*) and *S. non-scripta* (=*Endymion non-scriptus*) (Battaglia, 1958a), while it is of the Allium type in *S. amoena* (Tören, 1968) and *S. persica* (Svoma & Greilhuber, 1987). The Polygonum type is the most common type of embryo sac development among the angiosperms. *Chenopodium botrys* L. belongs to the angiosperm division, as does *Scilla autumnalis* L., and shows Polygonum-type embryo sac development (Chehregani et al., 2009). All cells in the embryo sac of *S. sibirica* are structurally organised so that synergids and egg cells are covered by a complete cell wall (Bhandari & Sachdeva, 1983). Recently, *S. siberica* subsp. armena was found as a new record (Haber & Semaan, 2007). The ploidy level in the antipodal cells of *S. bifolia* is 1024C DNA content, corresponding to 10 endoreduplication cycles (Nagl, 1976). In the family Liliaceae, the embryo development conforms to the Onagrad, Asterad, Caryophyllad, and Chenopodiad types. Endosperm development is of the nuclear or helobial type (Svoma & Greilhuber, 1987). This study aims to explore the structural and cytochemical features of the embryo sac, embryo, and endosperm development in *Scilla autumnalis*.

Materials and methods

The flower buds were collected at different developmental stages, in the Başıbüyük region of İstanbul, Turkey. The material was fixed in acetic-alcohol (1:3, v/v) at 4 °C for 24 h, and after dehydration in a graduated series of ethyl alcohol. Thereafter, the material was embedded in paraffin. The 6-10 µm thick microtome sections were stained with Regaud's haematoxylin. For cytochemical observations, sections were stained with Feulgen for chromatin (Yakar-Olgun, 1960), periodic acid-Schiff (PAS) for localisation of insoluble polysaccharides (Feder & O'Brien, 1968), Alcian blue (1% Alcian blue in 3% acetic acid) for acidic polysaccharides and pectins (Heslop-Harrison, 1979), and Coomassie Brilliant Blue (in a mixture of water, methanol, and acetic acid (v:v:v, 87:10:3)) for total proteins (Heslop-Harrison, 1973).

Nuclear volume was calculated according to the formula $4/3\ r_1r_2r_3\pi$ (Tschermak-Woess & Hasitschika, 1953). In large high-polyploid nuclei, the r_3 dimension represents the sum of values from successive microtome sections, and only nuclei of regular shape were calculated.

The preparations were analysed with Image-Pro Express software, assisted by an Evolution LC colour camera and an Olympus BH-2 microscope.

Results

In *Scilla autumnalis*, the mature ovule is anatropous, bitegmic, and crassinucellate. The inner integument initiates first, and then the outer integuments develop into a small protuberance. Integuments usually consist of 2 layers of cells. The micropylar part of the inner integument is composed of 4-5 cell layers at the stage of a mature embryo sac. Integuments show strong stainability for insoluble and acidic polysaccharides and contain numerous starch grains.

The nucellar epidermis is composed of one layer of cuboidal cells. The apical cells of the nucellar epidermis show radial elongation and form a 1- or 2-layered nucellar cap, the cells of which contain large vacuoles and a small amount of peripheral cytoplasm (Figure 1a). The nucleus is pushed to one side and usually has numerous nucleoli. The cell wall of the nucellar epidermis becomes thickened, increasing towards the micropylar region. The cells of the nucellar cap were stained strongly by PAS and Alcian blue. They are rich in acidic and insoluble polysaccharides, including numerous starch grains. Their cytoplasm appeared to be very rich in total proteins, as well. They secrete a PAS-positive exudate, filling the micropyle.

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 functional

megaspore stage. The numbers of cells increase in further stages and become more prominent in the 8-nucleate embryo sac. The hypostase cells are thick-walled, each containing dense cytoplasm and a prominent nucleus with a few nucleoli. The cytoplasm stained intensely for its insoluble and acidic polysaccharide content. Close to the antipodals, the hypostase cells stained more strongly, and they include abundant spherical starch grains (Figure 1b).

The obturator differentiates at the base of the funiculus as a small projection. It consists of thick-walled, greatly elongated, glandular cells. The nucleus of the obturator cells is rather massive. The cells of the obturator have numerous small vacuoles, fusing to form one large vacuole at the apical region in fully formed hypostase. The dense cytoplasm was strongly stained by PAS and Alcian blue (Figures 1c and 1d). The obturators include abundant starch grains and appear to be very rich in total proteins when stained by Coomassie Brilliant Blue, as well (Figure 1e). They secrete a surface exudate strongly stained by PAS. After fertilisation, the obturator cells shrank and disappeared.

A single archesporial cell differentiates as a megaspore mother cell (MMC), which undergoes meiosis to form a dyad and then a linear tetrad. Mitotic divisions in the functional megaspore result in 2-, 4-, and 8-nucleate embryo sacs (ES). The development of the embryo sac conforms to the Polygonum type. In a mature ES, the egg apparatus consists of 1 egg cell and 2 synergids. Polar nuclei lie somewhat below the egg apparatus, and they soon fuse to form the secondary nucleus. The secondary nucleus is close to the antipodals.

Egg Cell

The egg cell is polarised by the aggregation of cytoplasmic constituents at the chalazal end of the cell. The micropylar end is occupied by a large vacuole. The cytoplasm is present at the periphery of the cell and around the nucleus. It is rich in proteins and starch grains located in a perinuclear arrangement (Figures 2a and 2b). The egg cell is completely surrounded by a wall that is stained by PAS (Figure 2c).

Synergids

The 2 synergids are elongated cells and lie in contact with each other. They contain a large, chalazally located, uninucleolate, spherical nucleus. The cells are smaller than the egg cell. They are ephemeral structures and degenerate simultaneously soon after fertilisation. Their cytoplasm appears to be very rich in total proteins, as revealed by staining with Coomassie Brilliant Blue (Figure 3a). The nucleus and the nucleolus also stain densely for proteins. The synergids give a strongly positive reaction for insoluble and acidic polysaccharides and contain numerous starch grains (Figure 3b). The synergids are surrounded by a wall material that gives a PAS-positive reaction (Figure 3c). The filiform apparatus is the most conspicuous structure in the synergid and extends from the wall in the proximal portion of the cell up to almost its centre. It is a mass of finger-like projections of wall into the cytoplasm, stains intensely for insoluble polysaccharides, and comprises fibrillar projections (Figure 3c).

Central Cell

The nuclei of the central cell, the polar nuclei, fuse before fertilisation to form a secondary nucleus, which is located near the antipodals. The cytoplasm amassed around the secondary nucleus stained deeply for proteins. Numerous starch grains and PAS-positive granules were also present in the cytoplasm of the central cell, which was weakly stained by Alcian blue and PAS (Figure 4a).

Antipodal Cells

The antipodals are persistent uninucleate cells and have dense, darkly stained cytoplasm. They appear to be located in 2 arrangements at the chalazal end of the embryo sac. By linear arrangement of the antipodals, the embryo sac takes a long pouch shape and becomes oval by their triangular arrangement. A definite pectocellulosic wall completely surrounds the antipodal cell (Figure 4b). The cytoplasm has many small vacuoles and is highly rich in total proteins (Figure 4c). They contained abundant starch grains and PAS-positive granules, and they stained strongly for insoluble and acidic polysaccarides (Figure 4d). The antipodal nuclei showed a more intense Feulgen reaction when compared with the other cells of the embryo sac (Figure 4e). Antipodal cells persist up until the globular stage of the embryo, and then degeneration starts by the strong vacuolisation of cytoplasm, the collapse of cell walls, and the disintegration of nuclei.

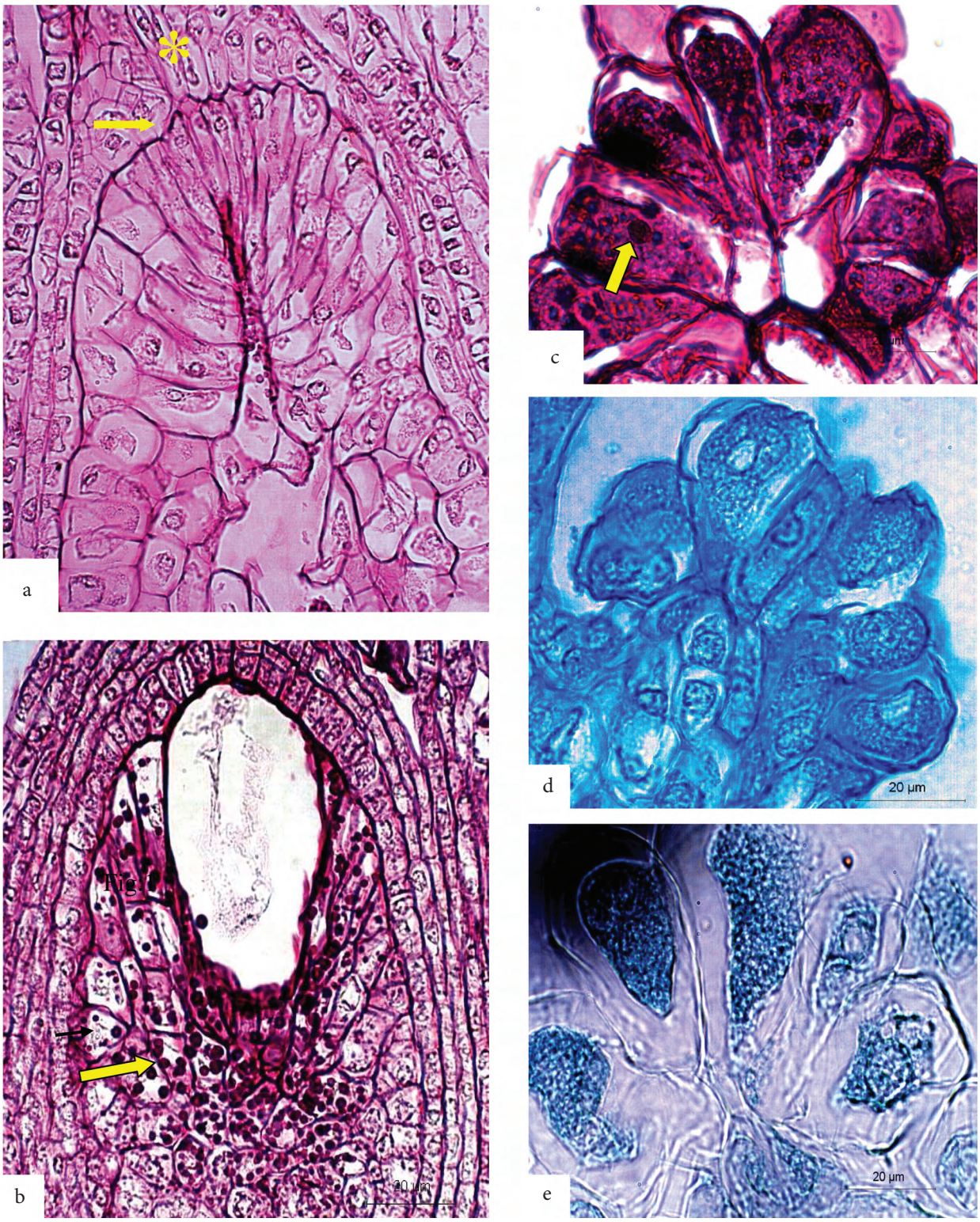

Figure 1. Ovular structures in *Scilla autumnalis*. (a) Nucellar cap (arrow) and micropyle (asterisk) stained with PAS. (b) Hypostase stained with PAS. Hypostase cells with numerous starch grains (arrow). (c) Obturator stained with PAS (arrow). Numerous starch grains in the cells of the obturator. (d) Obturator stained with Alcian blue. (e) Obturator stained with Coomassie Brilliant Blue.

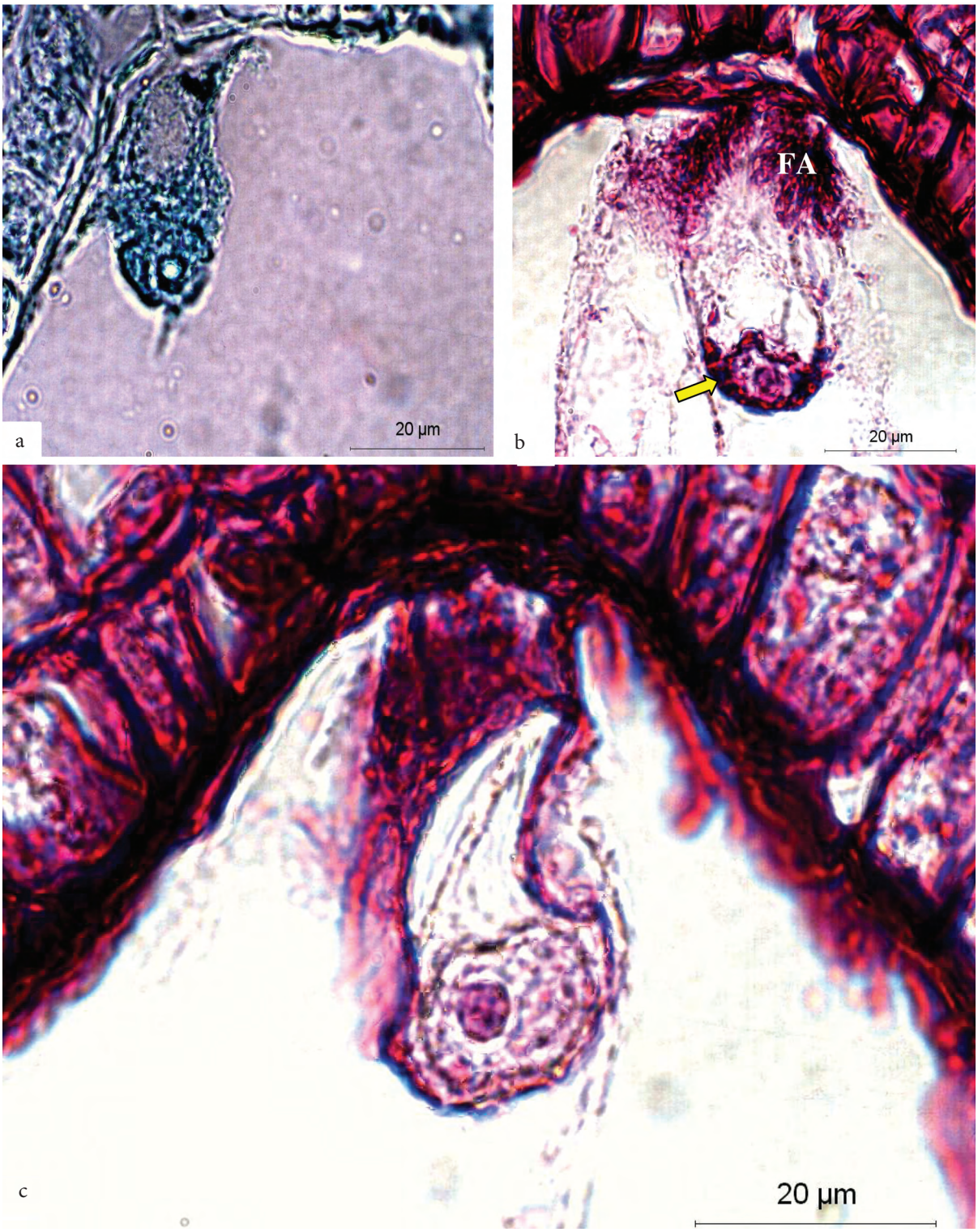

Figure 2. (a) Egg cell stained with Coomassie Brilliant Blue. Note that the nuclei and cytoplasm stain intensely for total proteins. (b) Egg cell stained with PAS. Note the presence of starch grains (arrow). (c) Egg cell illustrating the presence of a complete PAS-positive wall.

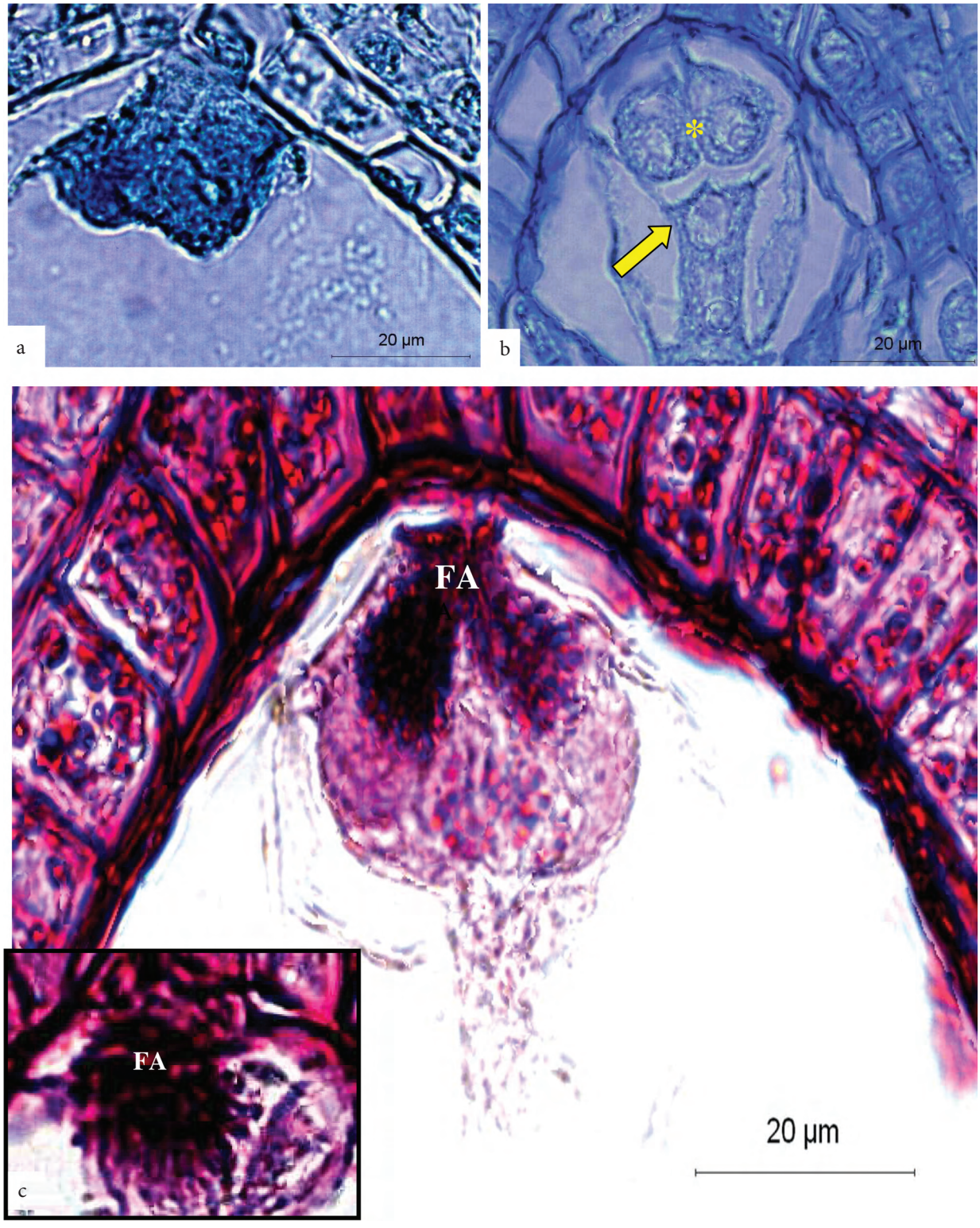

Figure 3. (a) Coomassie Brilliant Blue staining reveals high levels of proteins in synergids. (b) Synergids (asterisk) and egg cell (arrow). Alcian blue staining reveals the presence of acidic polysaccharides. (c) Two synergids illustrating the presence of a complete PAS-positive wall. Note the filiform apparatus and its magnified view at the bottom left. FA = filiform apparatus.

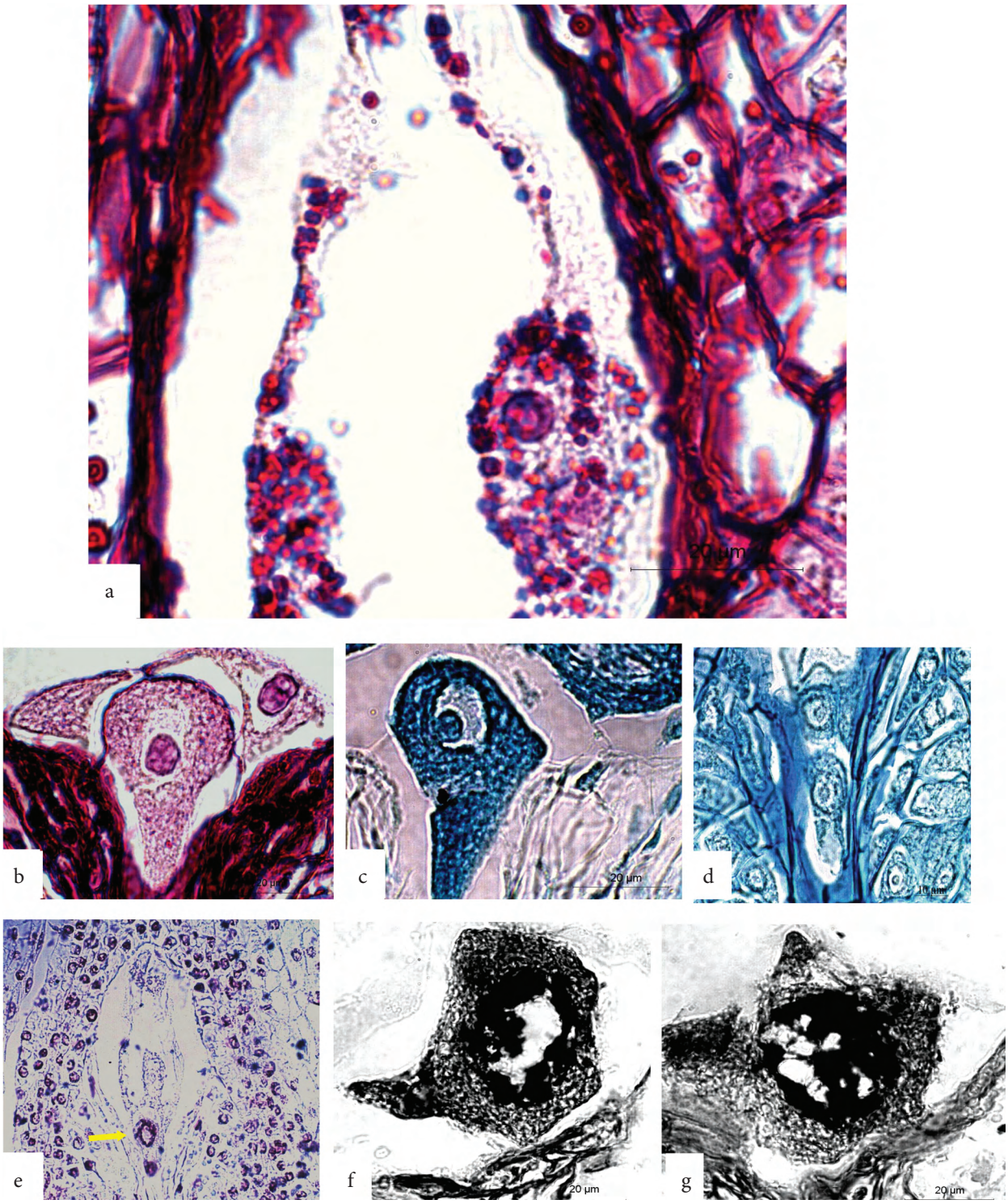

Figure 4. (a) Central cell stained with PAS. Note numerous starch grains. (b) PAS staining reveals the presence of a complete wall surrounding an antipodal cell. (c) Antipodals stained with Coomassie Brilliant Blue. (d) Antipodals stained with Alcian blue. (e) The antipodal nuclei (arrow) showed a more intense Feulgen reaction. (f and g) Different ploidy levels of antipodals that stained with haematoxylin. (f) An antipodal cell at 64n ploidy level. (g) An antipodal cell at 128n ploidy level.

The antipodals increased their volumes consequently with a considerable increase of the volumes of their nuclei. The nuclei of the antipodal cells are spherical or somewhat ellipsoidal. They are haploid only at the very early developmental stages, and then they undergo a rhythmical growth. The volume of the nuclei was calculated and the results are presented in the Table. The volume of antipodal nuclei ranged from 275,209 to 26,742,168 μm^3. From the calculations, 8 classes of nuclei, corresponding to ploidy levels n, 2n, 4n, 8n, 16n, 32n, 64n, and 128n, were established. The appearance of chromatin material suggests repeated chromosome endoreduplications.

Polyploidy levels in the triangular fashion of the antipodal cells were higher than the linear antipodals. In the latter, the ploidy level can rise to 8n as the maximum and is frequently at different ploidy levels of 3 antipodals, 2n, 4n, and 8n. The triangle-form antipodal cells, however, can rise to 128n, frequently at the same ploidy level of 3 antipodals, which represents the highest level of ploidy in this species (Figures 4f and 4g).

Embryo Development

Embryo development is of the Onagrad type. The zygote is smaller than the egg cell, resulting from the disappearance of the vacuole. The starch grains accumulate in the apical part of the zygote. The first division of the zygote was transversal, giving rise to a terminal cell and a basal cell almost equal in size (Figure 5a). The apical cell divides vertically to form 2 juxtaposed cells. The basal cell undergoes a transverse division to produce 2 superposed cells. Thus, a 4-celled proembryo was formed, its cells dividing in various planes to form a globular (Figures 5b-5f) and then a cylindrical embryo (Figures 5g and 5h). In the oldest, the cotyledon elongates (Figure 5g). At the mature cylindrical embryo stage, multicellular endosperm fills the embryo sac. The suspensor is short and usually consists of 2 or 4 cells. It disappears at the end of the development.

Endosperm Development

Endosperm is helobial in *S. autumnalis*. The primary endosperm nucleus is formed by the fusion of a sperm with the secondary nucleus, and it divides soon thereafter. The nucleus of primer endosperm divides at the level of antipodals to form a large micropylar and a small chalazal chamber. Many nuclear divisions occur in the micropylar chamber before it undergoes cellularisation (Figures 5i and 5j). By contrast, a few divisions occurred in the chalazal chamber and 6-10 nuclei remained coenocytic.

Cytochemical analysis indicated that the helobial endosperm shows a strong stainability for total protein and insoluble polysaccharides, as revealed by Coomassie Brilliant Blue staining (Figure 5i) and PAS reagent, respectively.

Table. Volume and degree of ploidy of the antipodal nuclei in *Scilla autumnalis*.

Degree of ploidy	Extreme values (μm^3)	Average (μm^3)	Number of nuclei
n	275,209.4-335,784.2	298,349.9	8
2n	528,510.9-637,522.9	588,743.9	9
4n	1,073,954.7-1,253,378.9	1,156,164.8	16
8n	2,103,580.2-2,465,203.6	2,215,052.1	14
16n	4,261,703.5-4,827,373.4	4,534,602.5	7
32n	8,416,805.3-9,982,169.4	9,082,928.7	9
64n	14,472,304.9-19,635,157.8	17,053,731.4	7
128n	23,412,388.3-26,742,168.5	25,064,170.3	4
	Total number of nuclei		74

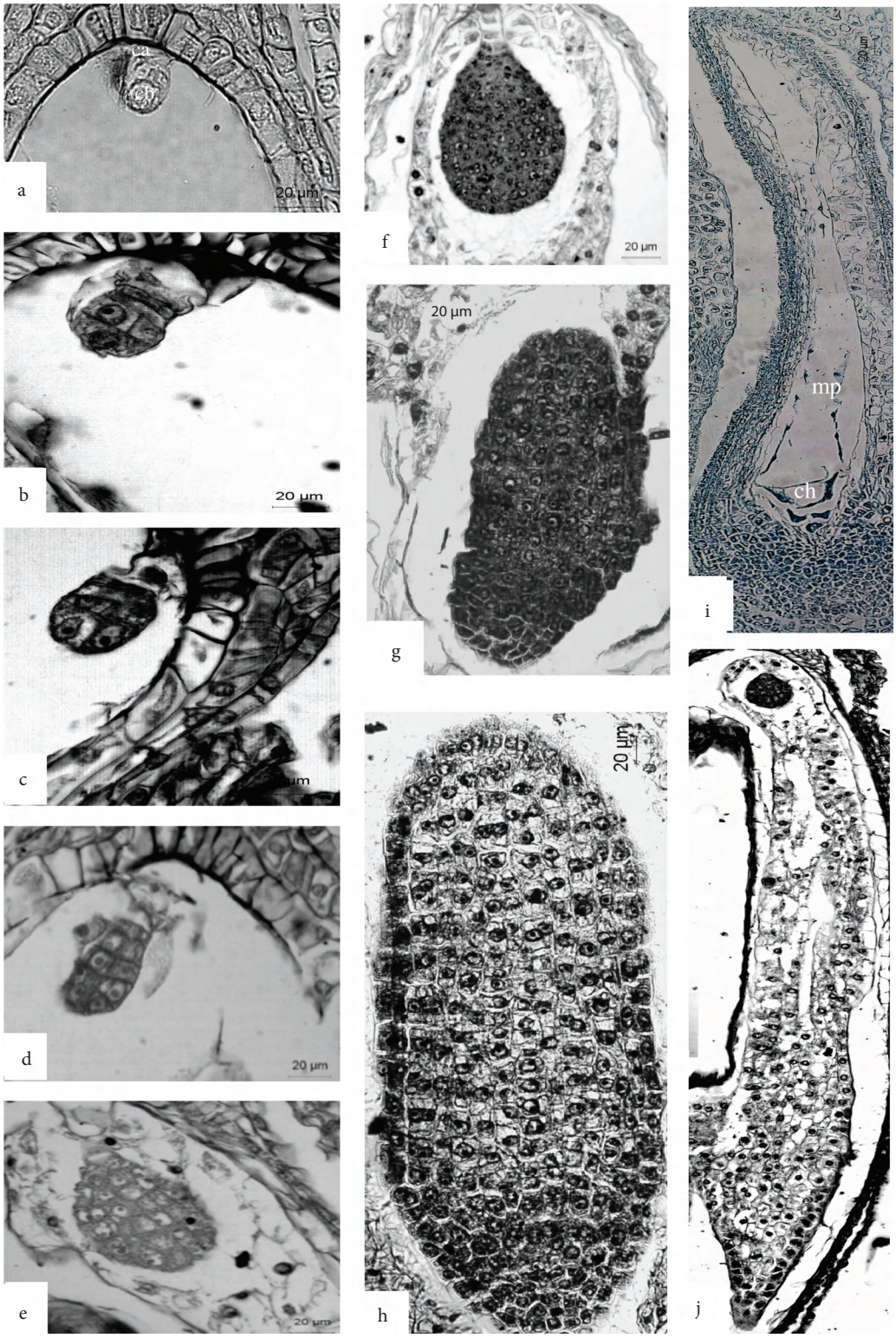

Figure 5. Embryo and endosperm development. (a) Two-celled proembryo; apical cell (ca), basal cell (cb). (b-d) Later stages of the proembryo. (e and f) Globular embryo. (g and h) Mature embryo. (i) Helobial endosperm; note the large micropylar chamber (mp) and small chalazal chamber (ch). (j) Later stage showing the cellular micropylar chamber and a globular embryo.

Discussion

In this study, we showed that the ovules of *S. autumnalis* are anatropous, bitegmic and crassinucellate. In addition, the mature embryo sac is monosporic and Polygonum-type.

In some taxa, the nucellar epidermis cells at the apex of the ovule become modified to form a nucellar cap (Tilton & Mogensen, 1979). The uniseriate cap was well exemplified in the family Agavaceae and in some members of Liliaceae, the multiseriate cap in other Liliaceae, the Poaceae, and the Cactaceae (Tilton, 1980a). It is thought the micropylar exudate is secreted by the nucellar cap in *Ornithogalum caudatum* (Tilton, 1980a). The cytochemical features of the nucellar cap in *S. autumnalis* suggest its secretory function that directs the pollen tube growth towards to embryo sac.

The hypostase cells show variation in structure; in *Delphinium camptocarpum*, the cells are surrounded by thick, laminated walls lacking plasmodesmata (Belyaeva, 1983). The hypostase cell walls may become lignified or suberised (Batygina & Shamrov, 1999). Their cell walls may also contain callose, cutin, and cellulose (Nath, 1993). In *S. sibirica*, the hypostase cells secrete substances of unknown composition, which diffuse in a fan-like manner (Grassimova-Navashina & Batygina, 1958) Depending on the plant species and the developmental stage, the hypostase cells can accumulate various storage reserves, such as proteins, fats, and tannins (Batygina & Shamrov, 1999). For instance, *Agave* normally accumulates carbohydrates, proteins, and fats, whereas only carbohydrates in *Ornithogalum caudatum* (Tilton, 1980b), and dextrins, starch, and proteins in *Gentiana cruciata* (Shamrov, 1990), are accumulated. Shamrov (1990) considers that the primary function of the hypostase appears to be supplying nutrients, and they are involved in the transport of metabolites to sporogenous and gametophytic structures.

Obturators occur in many taxa, especially in the families Euphorbiaceae, Rosaceae, and Liliaceae. It is secretory in *Ornithogalum caudatum* (Tilton & Horner, 1980), *Exospermum* (Sampson & Tucker, 1978), and *Drimys* (de Boer & Bouman, 1974). The chief function of the obturator is to guide the pollen tubes towards the micropyle.

The presence of large amounts of proteins and starch grains in the cytoplasm of the egg cell indicates that the egg cell of *S. autumnalis* is metabolically an active cell, as in *S. sibirica* (Bhandari & Sachdeva, 1983) and *Helianthus annuus* (Newcomb, 1973). This is contrary to the majority of observations (Schulz & Jensen, 1968; Van Went, 1970).

The well-developed filiform apparatus of *S. autumnalis* stained strongly with PAS, indicating its pectocellulosic nature, as was also shown in the synergids of *S. sibirica* (Bhandari & Sachdeva, 1983) and *Ranunculus sceleratus* (Vijayaraghavan & Bhat, 1980). Contrary to the majority of earlier observations (Jensen, 1974), the complete wall of the cells of the egg apparatus was also observed in *S. sibirica* (Bhandari & Sachdeva, 1983).

The central cell of *S. autumnalis* appears to be metabolically active, as in *S. sibirica* (Bhandari & Sachdeva, 1983). The hyperactivity of the central cell is related to the nutritive function of the endosperm.

In many species, the antipodals are persistent and show structural and cytological features suggesting their possible role in the nutrition of the embryo sac. Polytenisation has been described in the antipodals of many species, e.g., *S. biflora*, *S. sibirica*, *O. boucheanum, Narcissus pseudonarcissus, N. rotundifolia* (Hasitschka-Jenschke, 1962), *Epimedium pubigerum* (Ünal et al., 1997), and *Consolida regalis* (Ünal & Vardar, 2006)

Onagrad-type embryo development (Natesh & Rau, 1984) and helobial-type endosperm development (Vijayaraghavan & Prabhakar, 1984) are characteristics of the family Liliaceae. Cytochemical tests indicate the presence of high amounts of proteins and polysaccharides in the endosperm of *S. autumnalis*, as in *Allium peroninianum* (İsmailoğlu et al., 2006).

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