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Study of seed coat microsculpture organization during seed development in *Zygophyllum fabago* (Zygophyllaceae)

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Abstract: The ontogeny of seed coat and endosperm tissue in *Zygophyllum fabago* L. was studied to determine their developmental importance using different histochemical and microscopic techniques. Our results revealed that the ovule of *Z. fabago* was of the anatropous and bitegmic type. The inner epidermis cells were retained up to the end of seed development, whereas the other layers were removed in the early stages. Moreover, the outer integument was changed into the seed coat sculptures during the seed development. Concurrently, multiple cytoplasmic strings were formed at the seed coat cells. Fluorescence microscopic analysis indicated that callose and polyphenols were laid down at these strings. In the late stages of the seed development, the nucleus and cytoplasm of the cells were degenerated and the sculptures became obvious on the seed coat. The seed coat sculptures may play a role in the seed dispersal by wind. During the early developmental stages, the endosperm was of the nuclear type and then changed into the cellular type. Cytochemical tests indicated that in the later stages of seed development, the formation of starch grains and the thickening of cell walls occurred, causing considerable reduction of cell cavities as well as hardening the tissue. The cell storage in the endosperm tissue was more lipid-based than protein-based. Generally, due to the degeneration of the outer integument and the existence of the thin inner integument, the endosperm cell wall seemed to be thickened to protect the embryo and to save carbohydrates. The obtained results shed more light on the development of seed tissues in the family Zygophillaceae.

Key words: Bitegmic, exotesta, embryo, histological techniques, nuclear endosperm

1. Introduction

Zygophyllaceae as a heterogeneous family was divided into 5 subfamilies which comprise 27 genera and 285 species (Zhang et al., 2013). One of the biggest subfamilies is Zygophilloideae, which consists of taxonomically important genera including *Zygophyllum*, *Roeper*, *Melocarpum*, *Fagonia*, *Augea*, and *Tetraena* (Bellstedt et al., 2008). Just about 51 species of the genus *Zygophyllum* were reported in Asia. Members of the genus *Zygophyllum* were adapted to drought and heat stress conditions and found largely in Mediterranean regions (Wu et al., 2015). *Zygophyllum fabago* L. (*Z. fabago*), commonly known as Syrian bean-caper, is a perennial herbaceous plant, which is also famed as "Memeli Uzerlik" in the Azerbaijan region of Iran. In the folk medicine of Iran, this species has been named as "Qeich," with recognized anthelmintic and cathartic properties (Yaripour et al., 2017). The vegetative parts have been reported to possess antitussive, antifungal, antiasthmatic, expectorant, antiinflammatory, antibacterial, and antirheumatic effects and were externally used as remedy for wounds, injuries, and skin diseases (Khan et al., 2014). The plant possesses long, thin stems with few fleshy leaflets. Flowers are small and appear on short stalks. Each has 5 green sepals and 5 petals that are white to cream with salmon-colored markings. Ten orange stamens extend past the petals. Each flower has an ovary with 4–5 locules encompassing ovules which are often of the anatropous type, developing flat and rough seeds (Erdemoglum and Kusmenoglu, 2003; Semerdjieva and Yankova-Tsvetkova, 2017).

Morphology, color, size, structure, and composition of the seed coat, the nature of seed storage components, and the presence or absence of endosperm are valuable information on seed characteristics of plant species (Patil et al., 2015; Takahashi et al., 2016; Umdale et al., 2017). The extent and structure of endosperm are diverse in mature seeds. Proteins are known as one of the important storage materials in the endosperm (Lersten, 2004). The other important storage components are lipids, which claim the majority of storage contents of oil seeds. Additionally, mineral materials and carotenoids are accumulated in the endosperm (Lersten, 2004).

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Variable seed coat traits are known to be cell size and shape, formation of papillae, and periclinal/anticlinal cell wall orientation (Galek et al., 2016). Primary superficial sculptures are related to the shape and arrangement of epidermis cells of the seed coat (Oriani and Scatena, 2014). Different types of cuticle contents, as well as the papillae of external walls of epidermal cells, establish secondary superficial sculptures (Salimpour et al., 2007). The micromorphology of the wax on the cuticlular layer and its sedimentation characteristics create tertiary superficial sculptures (Salimpour et al., 2007).

The sculpture-based patterns of the seed coat were used for determining adaptation characteristics of the seed coat (Zeng et al., 2006). During the seed development, the seed coat shows significant histochemical changes, which are related to its multiple functions (Queiroz et al., 2013). Many seeds are characterized by sculpturing structures, which are bands of lignified thickening in the walls of the testa layer. Lateral strands probably contain a combination of lignin and cellulose, and the bases consist of pectins. Generally, the thickening material is composed of cellulose, lignin, pectin, and suberin (Zeng et al., 2006).

By determining different morphological characteristics of the seed coat, many genera were classified in separated families (Behnke et al., 2013; Nath and Dasgupta, 2015). Furthermore, endosperm as a nutritional tissue in flowering plants shows substantial cytological changes during ontogenetic stages. Thus, morphological and anatomical study of the seeds may suggest a number of taxonomical diagnostic characters for distinguishing genera and species of heterogeneous families such as Zypophyllaceae. *Z. fabago* is a widespread species, and its flowering stage is relatively long (from late spring up to about the end of summer) (Ghazanfar and Osborne, 2015). Each ovary contains a high number of seeds (about 40); therefore, this species seemed to be suitable for the aims of the current research. The objective of the current work was to study the development of endosperm tissue and seed coat of *Z. fabago*. Accordingly, the general composition of cell layers in seed coat was clarified. The findings of the present work provide a basis for further developmental and cytochemical studies on the seed of the members of Zypophyllaceae.

2. Materials and methods

2.1. Sample collection and processing

Plants (*Z. fabago*) in different developmental stages (from nonopened blossoms up to mature seeds) were collected for histological, cytochemical, and developmental studies from the campus of Tabriz University in late spring, 2017.

2.2. Preparation of whole mount samples

In order to determine the space occupied by the embryo in the seed, the seeds were initially treated with a 0.5% NaOCl solution at 60 °C. After destaining, they were immediately transferred into distilled water. They were subjected to a solution of 97% alcohol for 15 min and then were transferred into an alcohol-xylol solution. Finally, the samples were examined by means of a SMZ 1500 Nikon stereomicroscope and were photographed by a Canon digital camera (Ilarslan et al., 2001). In order to capture images of all the samples at the suitable depth of field, we set out to secure a series of images where the first had the highest focal plane and each consecutive image had an incrementally smaller focal plane. These images were then imported to ImageJ 1.41 software (http://rsb.info.nih.gov/ij/), and depth of field was enhanced automatically using the method reported by Movafeghi et al. (2010).

2.3. Microscopic analysis

The samples (seeds and ovaries) were fixed in alcohol-formalin-acetic acid (17:2:1, v/v/v) and formal-calcium (10:5, v/m) for 24 h, dehydrated in an alcohol series, cleared in toluene, and embedded in paraffin. Sections 8–10 µm thick were obtained by using a rotary microtome. Then, the slides were cleared in toluene, rehydrated in an alcohol series, and stained.

Recognition of polysaccharides was performed according to the Periodic acid–Schiff (PAS) staining protocol (Jensen, 1962). The staining was done by hematoxylin, light-green and toluidine blue 2% (Gahan, 1984). To identify pectin by red ruthenium solution, the samples were prepared according to Jensen's (1962) method. The identification of proteins was performed by the Coomassie Brilliant Blue g-250 staining method (Gahan, 1984), and the identification of phenolic components was performed by implementing Gutman's (1993) method. The identification of callose (Jensen, 1962) and suberin (Gahan, 1984) was performed by using a Nikon Eclipse E1000 fluorescence microscope.

Scanning electron microscopic (SEM) analysis was conducted using the protocol reported by Terziyski (1981). Finally, the samples were examined using a Zeiss LEO 435VP SEM at 20 kV.

3. Results

3.1. Ontogeny of the seed coat

The study of the cleared seeds of *Z. fabago* revealed that the embryo is of spatulate type and it occupies around ¾ of the seed capacity. The mean size of the seeds was approximately 4.5 mm (Figure 1). The anatropous ovule of *Z. fabago* was recognized as bitegmic. The outer integument was composed of 2 layers of cells with thickened periclinal walls, a dense cytoplasm, and a large central nucleus. The cells of the inner layer of the outer integument contained starch grains, which were detected with PAS reagent (Figure 2a).

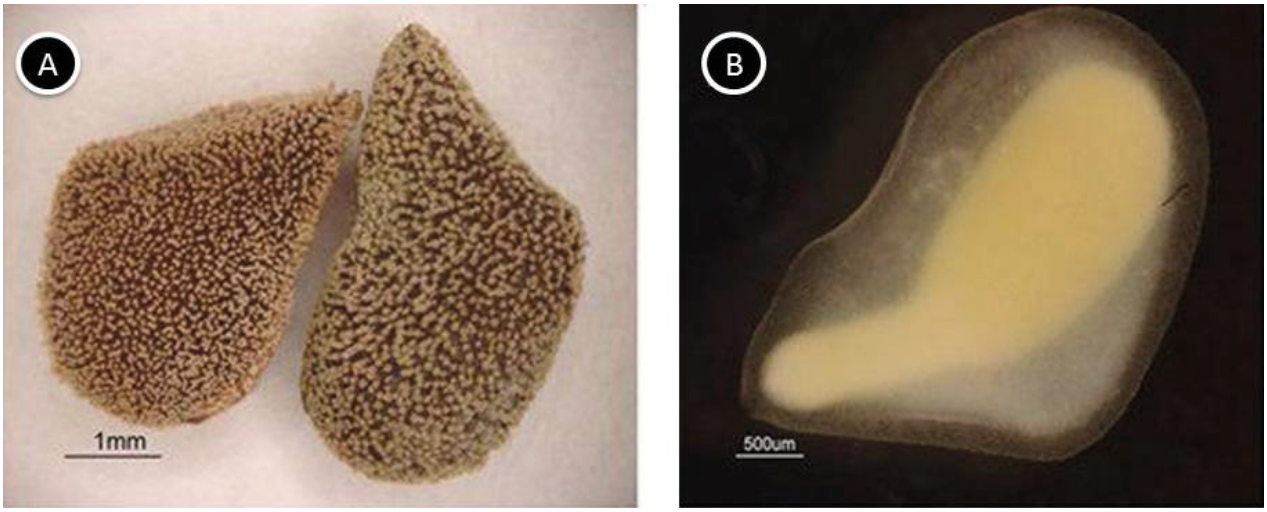

Figure 1. General view of the seed of *Z. fabago* taken by a stereomicroscope. A, Abundant superficial sculptures in the coat. B, The volume occupied by the embryo in the cleared seeds.

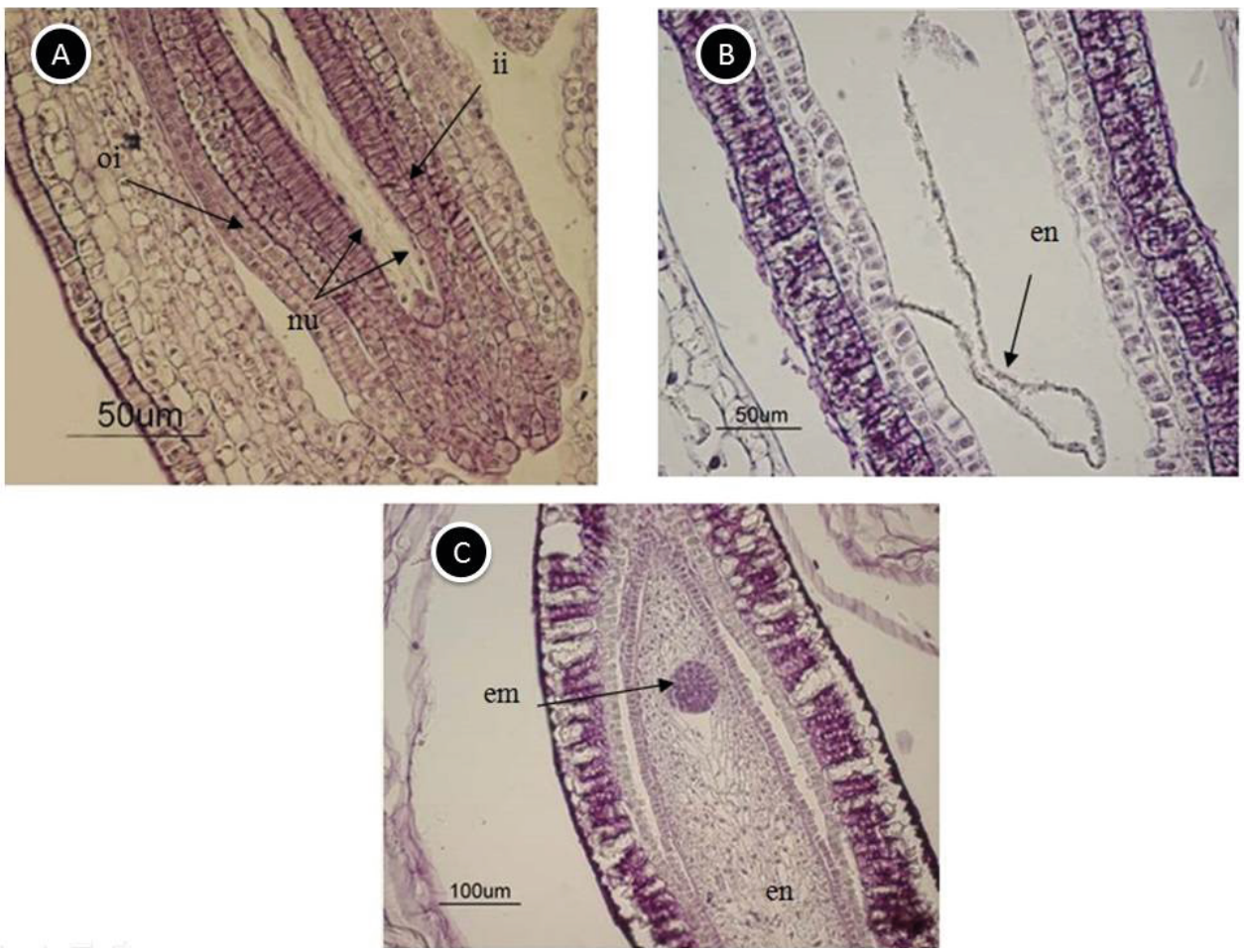

Figure 2. Longitudinal section of the ovule stained by PAS-hematoxyline method. The bitegmic anatropous ovule of *Z. fabago* is observable. A, Ovule integuments. B, Free nucleus bodies of endosperm in a linear arrangement. C, Filling the embryo sac by endosperm cells during the globular embryo stage. Initiation of the formation of the seed coat sculptures was observed. ii: inner integument, oi: outer integument, em: embryo, en: endoderm, nu: nucleus.

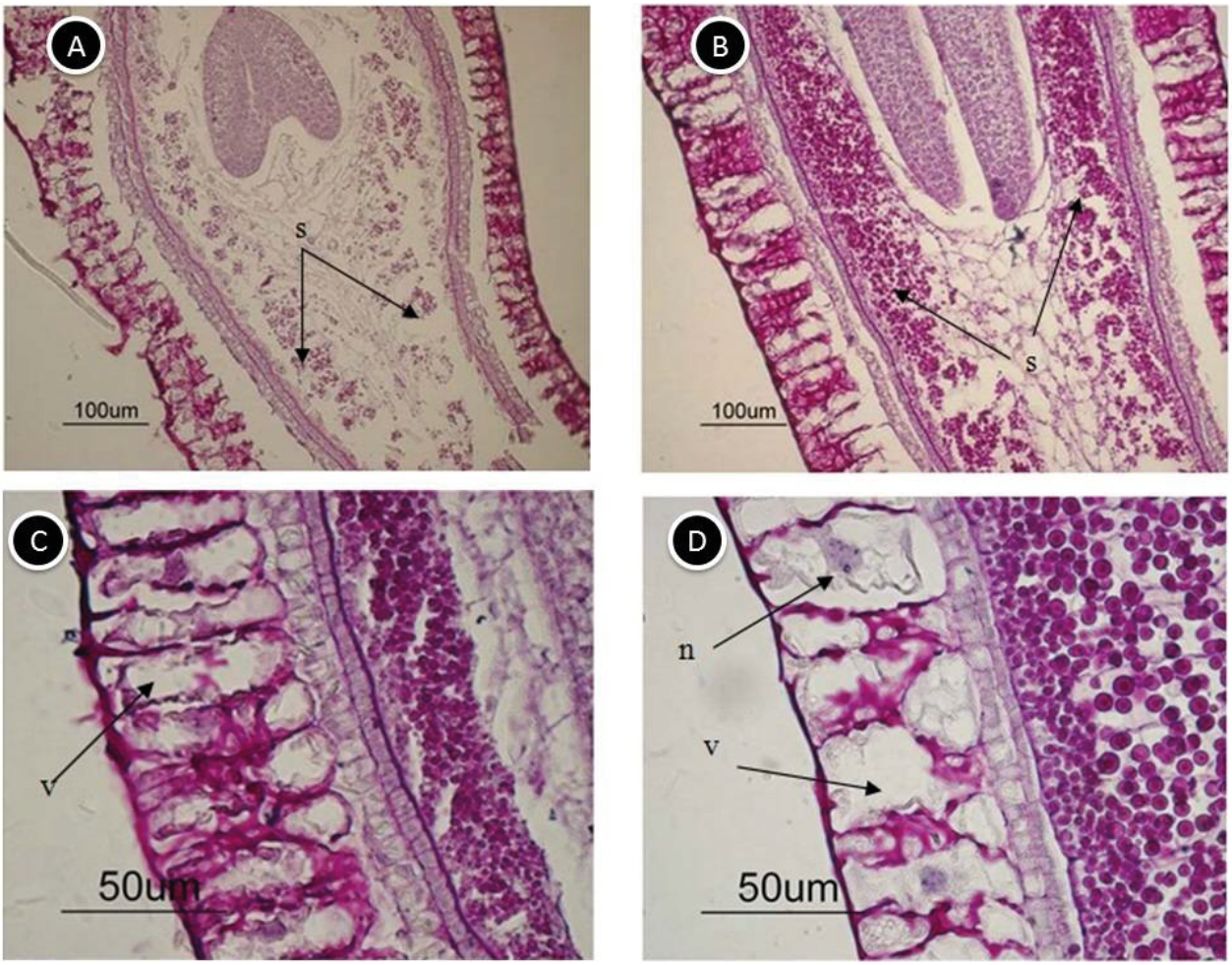

Figure 3. Longitudinal section of the ovule stained by PAS-hematoxyline method. A, Heart-shaped embryo stage in which the endosperm cells of the starch grains appeared. B, Torpedo-shaped embryo stage in which increasing of the starch grains in endosperm cells was observed. C, External epidermis of the seed with large vacuoles. Ripple formation of membrane and distance creation were obvious in the voluminous cells. D, Remarkable increasing of the volume strongly and shrinkage of cytoplasm in a number of cells. s: starch grains, v: vacuole, n: nucleus.

The inner integument was comprised of 3 to 4 distinct cell layers around the embryo sac. They may play a role in the formation of micropyle. The starch grains were observed in the outer integument cells. In comparison, the inner integument cells lacked the starch grains. The remaining nucellus tissue cells were observed in the marginal parts of the embryo sac (Figure 2a).

When the ovary developed further, the size of the ovules became enlarged. During these stages and the endosperm development, the ovule integument layers changed gradually (Figure 2b). During the globular embryo stage, complete filling of the embryo sac with endosperm cells and initiation of the formation of the seed coat sculptures were observed (Figure 2c).

In the heart-shaped embryo stage, the formation of various starch grains in the endosperm cells was observed from the margin to the center (Figure 3a). The number and the size of starch grains were significantly increased in endosperm cells (Figure 3b).

Ripple formation of membrane and distance creation were observed in the voluminous cells (Figure 3c). In many exotestal cells, volume enlargement occurred. Also, some cells remained the same as the number of the primary forms (as slender and stretched). All of these cells were alive and had an obvious nucleus. However, vacuolization had commonly occurred in these cells (Figure 3d).

3.2. Seed coat sculptures

During the globular embryo stage, volume increase of the coat cells and fracture of the primary cell walls were significant. This process caused the cell walls to be observed as considerably rough and irregular. Because of the special shape and kind of extensions of the seed coat cell

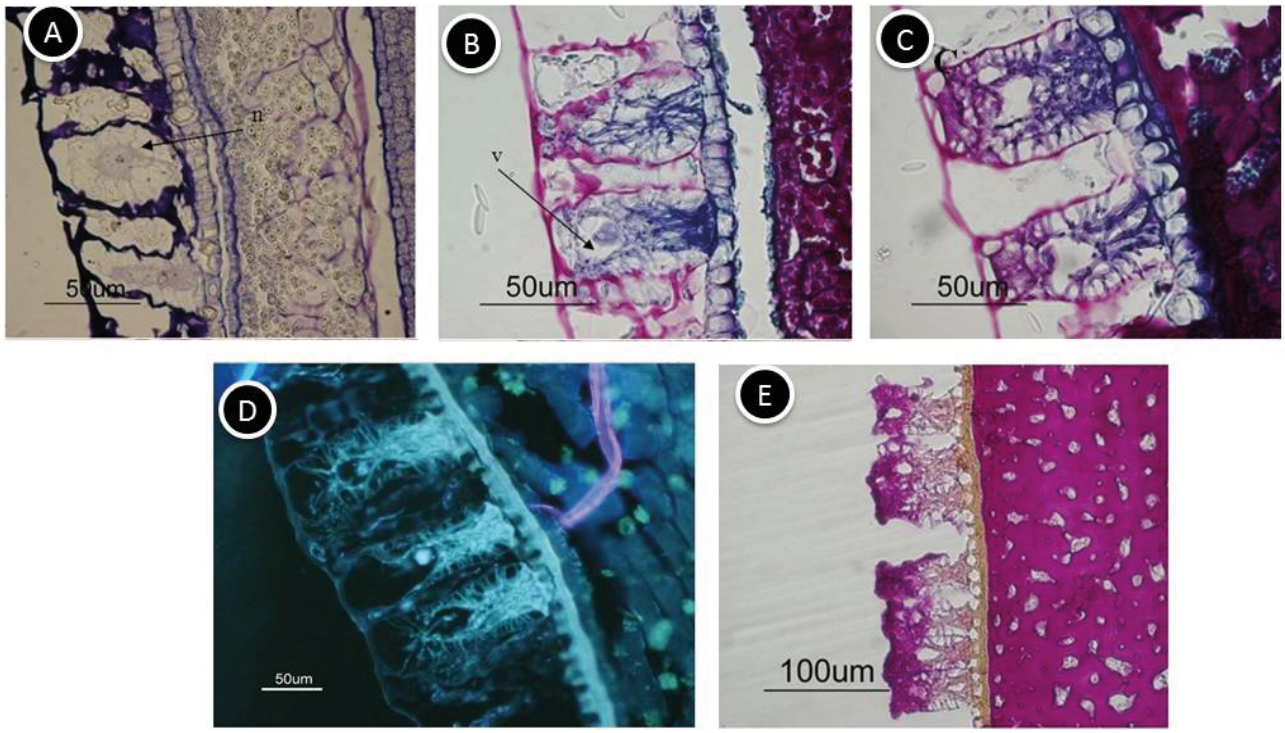

Figure 4. Longitudinal section of the ovule. A, The exotesta layer stained with Toluidine Blue. B, Cytoplasm has found a new rearrangement and abundant strings were formed. The exotesta layer stained with PAS-light green. C, The nucleus degeneration has occurred in most cells. The exotesta layer stained with PAS-light green. D, Longitudinal section of the seed sculpture after callose-specific fluorescence microscopy assessment. The filamentous structures appeared as branches of the secondary cell wall. E, The sculptures of the seed stained with PAS-hematoxyline. v: vacuole, n: nucleus.

walls, the cytoplasm seemed fractional. Following the seed development, multiple cytoplasmic strings were formed in a particular arrangement in these cells (Figure 4a). During the late stage, the cytoplasm has found a new rearrangement and abundant strings were observable inside the cells. They were gradually wrapped around the nucleus as interwoven structures (Figure 4b). In order to identify the nature of the strings, different cytochemical tests were performed. Using the PAS-light green protocol, the strings were seen in dark-blue color. However, there was no reaction with red ruthenium and safranin as specific stains for pectin and lignin, respectively (Figure 4b).

During the final stages of the seed sculptures development, the nucleus degeneration occurred in most cells (Figure 4c). Callose deposition in the cell wall associated strings was confirmed by fluorescence microscopy, but suberin was not detected. Taken all together, it seems that the interwoven strings included both polysaccharides and polyphenols (Figure 4d).

In the mature seeds, the nucleus and cytoplasm were totally removed and the internal space of the cells was filled by the dense and interwoven strings. These filamentous structures appeared to be branches of the secondary cell wall (Figure 4e).

Scanning electron microscopy images showed that the mean seed size was around 4.5 mm (Figure 5a). The cross-sectional surface area of each seed coat sculpture was 45 µm and its length was about 110 µm. There was some space between a number of voluminous cells, forming possibly because of the degeneration of slender and stretched cells (Figures 5b–5d).

3.3. Endosperm ontogeny

After fertilization, free nuclei of endosperm were observable in the embryo sac. In this stage, only a small amount of cytoplasm encompassed free nuclei. Therefore, the endosperm development was considered as the nuclear type. Indeed, although the division of free nuclei of endosperm began faster than the zygote, it seemed that mitotic divisions continued slowly. Then, the nuclei moved to the margin of embryo sac and were placed regularly. In this stage, the endosperm still seemed to be of the nuclear type (Figure 6a). In the next stage, the cell walls were formed between free nuclei (Figure 6b), and endosperm cellularization gradually occurred from the micropyle pole (Figure 6c). Finally, in the globular embryo stage, the embryo sac space was completely filled by endosperm cells. Following the development of the embryo to reach the torpedo-shaped stage, the starch grains emerged

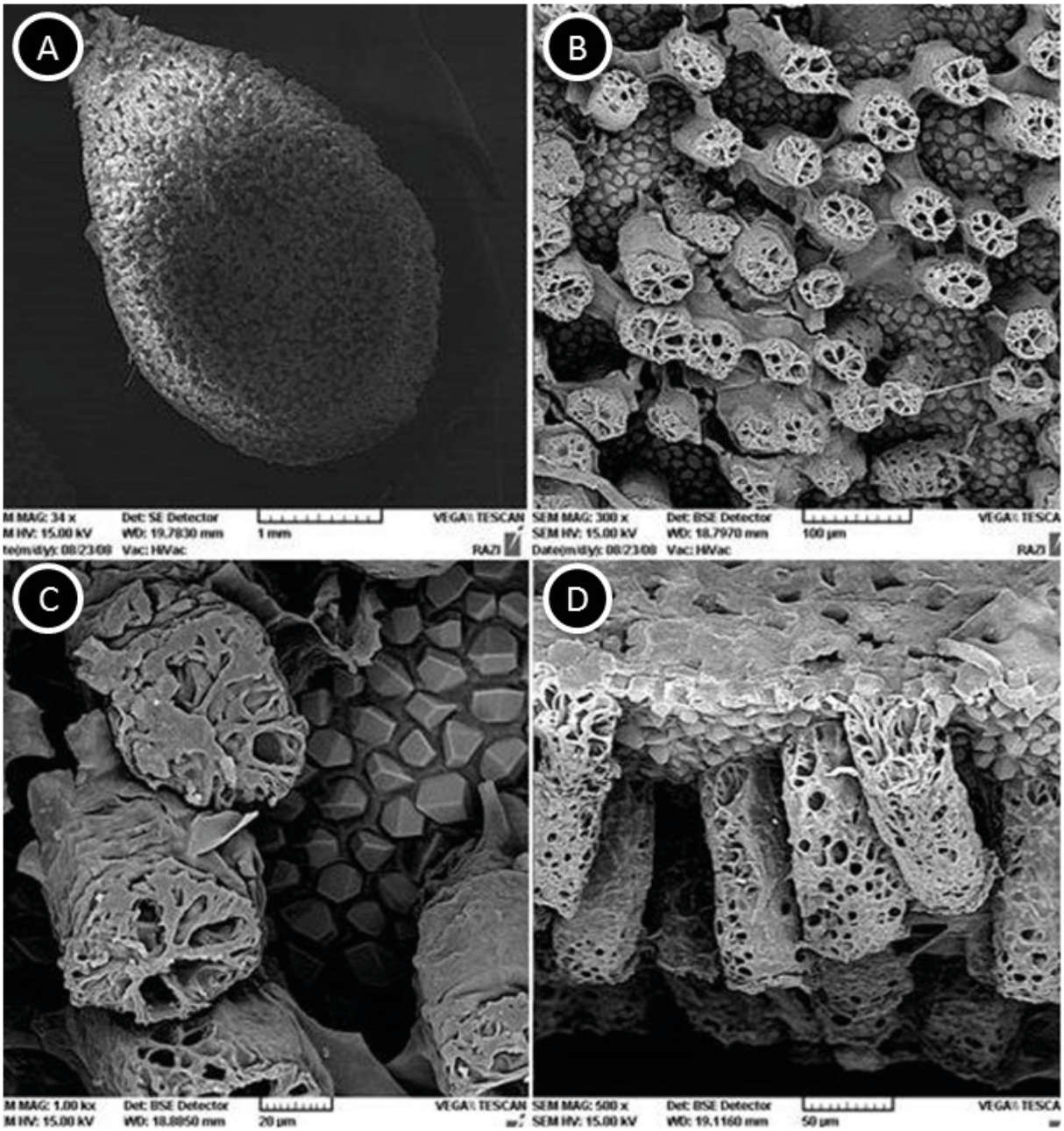

Figure 5. Ultrastructure of the seed coat sculptures. A, Mature seed. B–D, Sculptures of the seed coat. There was some space between a number of the voluminous cells.

in the endosperm cells. The starch grains in the stage of mature embryo were so abundant that the boundary of the endosperm cell walls was hardly recognizable (Figure 6d). Then, green and soft seeds with large starch grains inside the cells were produced. Definitely, the first layer of the endosperm has smaller cells and smaller starch grains compared with other layers (Figure 6e).

When the seed was yellow, thickening of the endosperm cell walls was maximized. The significant change during the late stages of endosperm development was the formation of asymmetrical cell cavities. Cytochemical tests indicated that the cell storage in the mature seed was more lipidic than proteinaceous (Figure 7).

3.4. Embryogenesis

The cellularization of endosperm and thickening of internal and external cell walls of the seed coat were observed during the globular embryo stage. Following the embryo development and reaching of the torpedo-

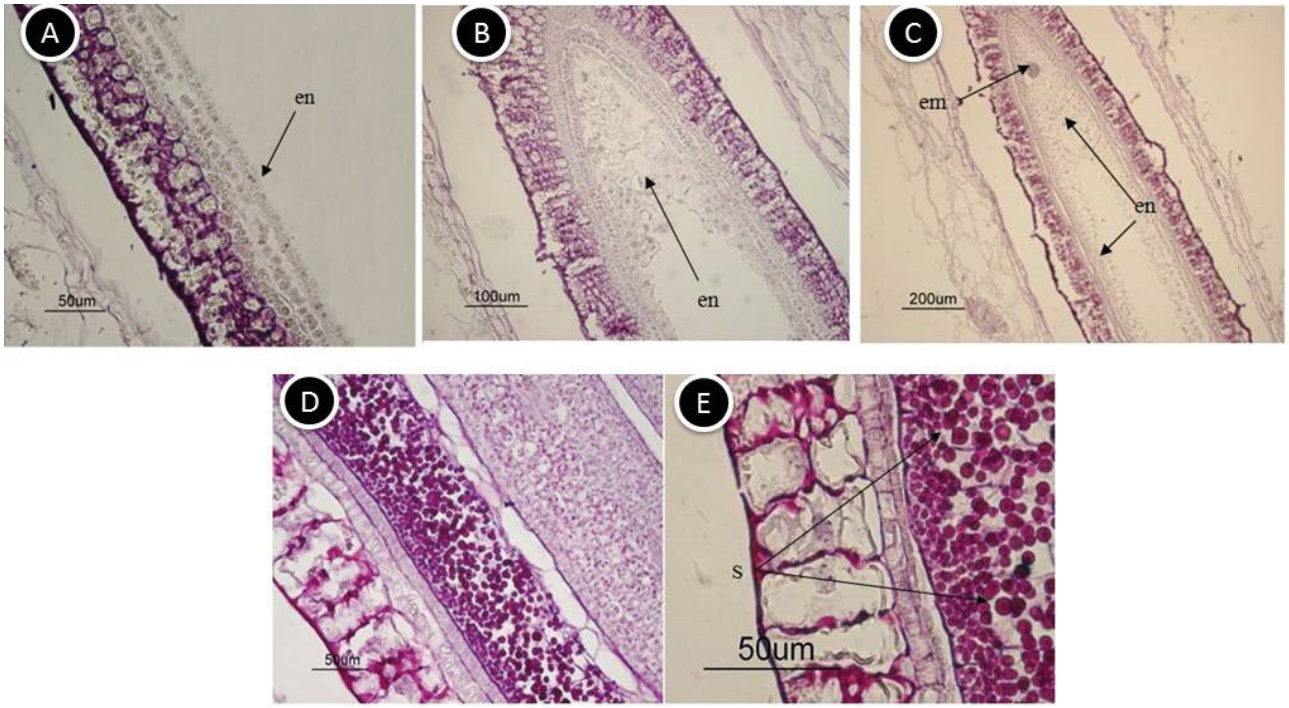

Figure 6. Ontogeny of endosperm. A, Migration of free nucleus bodies to the margin of embryo sac. B, C, Increased endosperm cells in micropyle pole. D, E, Increase in the level and size of starch grains (S), respectively, in the endosperm cells. en: endosperm, em: embryo.

shaped embryo stage, the starch grains in the marginal layer had a smaller size compared with the starch grains of other parts of the endosperm tissue (Figure 8a). By formation of elongated cotyledons and differentiation of apical meristems, the embryo axis was completed. Large central cells of the endosperm tissue were replaced by the developed cotyledons and encompassed the endosperm tissue (Figure 8b). Therefore, the embryo occupied a great portion of the seed space (Figures 8b and 8c). All cells were still full of starch grains. The size of starch grains was increased in comparison with the torpedo-shaped embryo stage. The starch grains were recognized in the embryo cells in this stage for the first time, but their quantity and size were lower than the endosperm starch grains. In this stage, the cotyledons reached their maximum growth, but the amount of starch grains decreased. The mature embryo in *Z. fabago* had a relatively long hypocotyl and large cotyledons. The embryo had a green color up to the cotyledonary stage and could be regarded as a "Chloroembryophyte" (Figure 8d).

4. Discussion

The shape of the seeds in *Z. fabago* was recognized as oval-rhomboid. The seed coat was covered by abundant superficial sculptures. The anatomical and ontogenetic characteristics of seed microsculptures could be used for taxonomic identification of plant species (Schenk et al. 2013; Szkudlarz and Celka, 2016). According to previous morphological and ultrastructural studies of the seed coat (Fredes et al., 2016), the seed coat sculpture development in *Z. fabago* could be divided into the following stages:

1- Longitudinal growth and elongation of the exotestal cell walls in anticlinal orientation, 2- Formation of multiple lamella as a result of the primary wall development in these cells, 3- Rapid increase in cells' volume and fracture of lamella, 4- Formation of multiple and interwoven strings of the secondary cell wall in the internal space of the cells, 5- Degeneration of nucleus and cytoplasm of the exotestal cells simultaneously with stage 4.

The endotegmen may commonly contain mucilaginous substances, whereas the exotegmen represents cell wall thickening (Sousa-Baena and De Menezes, 2014). In exotestal cells of *Z. fabago*, it is assumed that programmed cell death (PCD) occurs with a central developmental purpose. Accordingly, these cells will achieve their specific function after death. Different evidence, including shrinkage of cytoplasm, nucleus transformation, formation of multiple nucleoli, and transformation of cell walls during different stages reinforce this concept (Nishawar et al., 2008). The existence of such sculptures in the seed coat serves as air sac; therefore, it seems that they are well-matched with the seed dispersal strategy of the plant species (Tsou and Mori, 2002; Batygyina, 2006).

One of the other important changes in the seed coat was the emergence of starch grains and their consequent depletion during the mature embryo stage. Starch grains

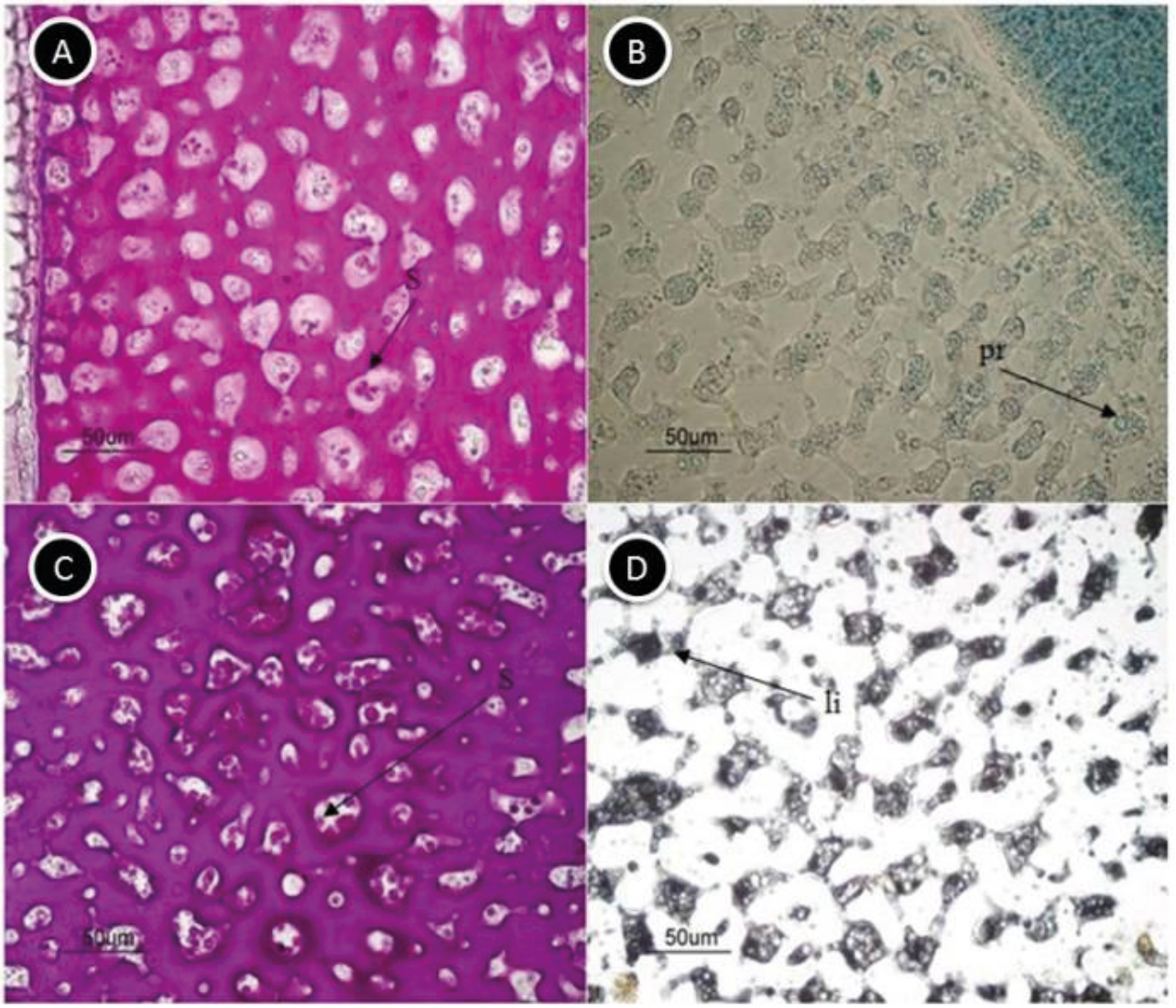

Figure 7. Identification of storage materials of endosperm. A, Increase in thickening of endosperm cell walls stained by PAS-hematoxyline method. B, Proteins were stored in the endosperm cells stained by Coomassie Brilliant Blue method. C, Increase in thickening of endosperm cell walls stained by PAS-hematoxyline method. D, Lipids were stored in endosperm cells stained by Black Sudan method. Pr: proteins, S: starch grains, li: lipid.

in the seed coat cells were initially known as precursors for the mucilage synthesis. Their presence before the mucilage secretion and their depletion during the seed maturity are in fair agreement with this concept (Moise et al., 2005). However, 2 major reasons contradicted this scheme. First, some *Arabidopsis* mutants were identified which were deficient in the starch metabolism, but had normal levels of mucilage. Second, in some cases the production of mucilage was reported to be performed before the hydrolysis of the starch grains (Moise et al., 2005). In the seeds of Z. *fabago*, the starch grains appeared to be precursors of cell wall's polysaccharides, since there is close time conformity between the destruction of the starch grains and formation of the primary cell walls in exotestal cells. Another option is the absorption of components resulting from the hydrolysis of starch grains by the developing endosperm and embryo. The function of polysaccharide composition was suggested to be a supplier of nutrients for the developing embryo (Voiniciuc et al., 2015). However, concerning the developmental characteristics of embryo and endosperm in *Z. fabago*, it seemed that the starch grains of the seed coat do not play a role in controlling the development of embryo.

According to the histological analysis, the endosperm development in Z. *fabago* is of the nuclear type. When the zygote fails to divide, endosperm cells perform rapid

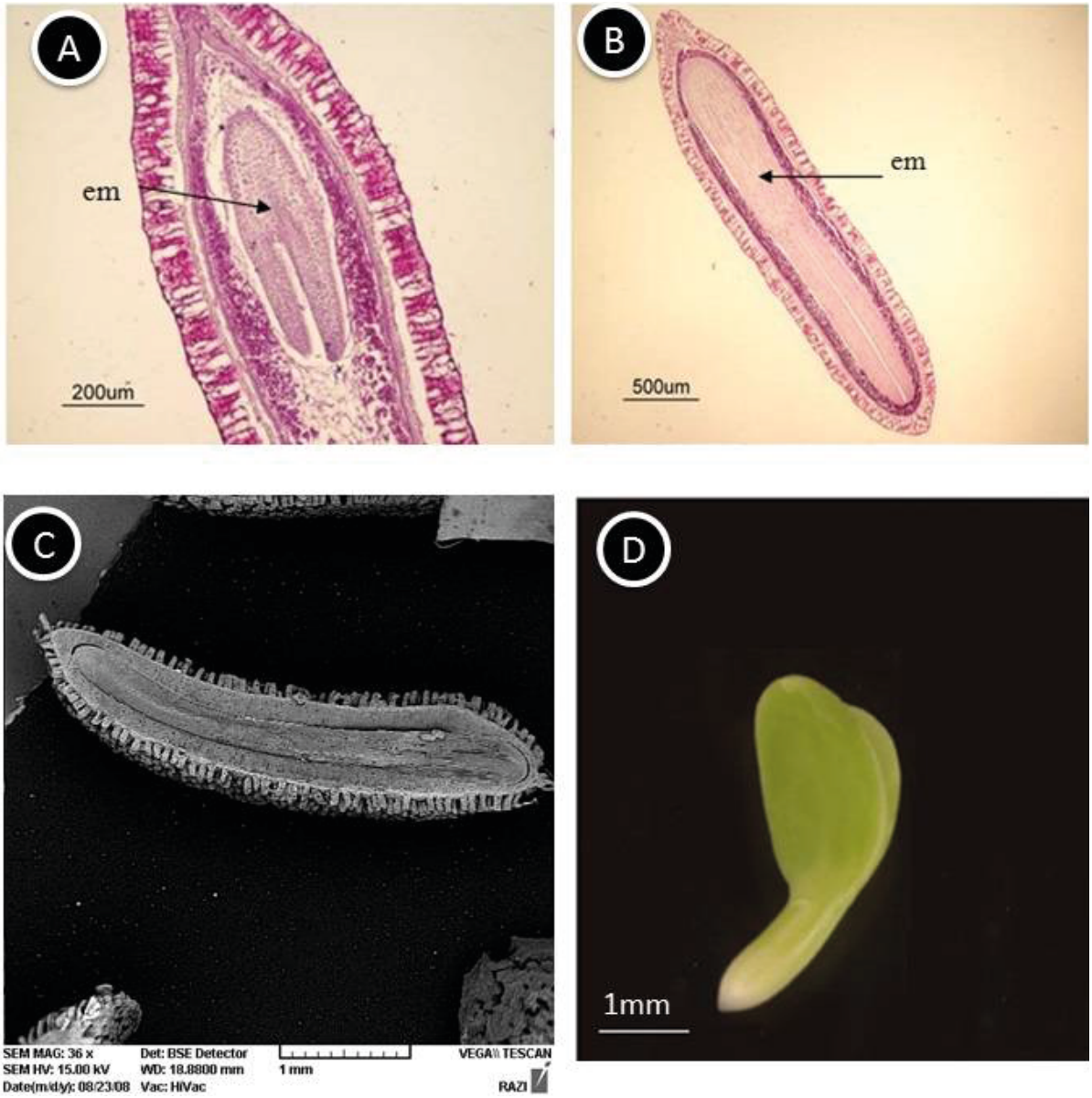

Figure 8. Final stages of embryogenesis. Large central cells of the endosperm tissue were replaced by the developed cotyledons and encompassed the endosperm tissue. A, Torpedo-shaped embryo. B, Growth and development of cotyledons. C, Scanning electron microscope image of a halved seed. D, Chloroembryophyte. em: embryo.

mitotic divisions, and a number of free nuclei are produced in the cytoplasm of central cell (Lersten, 2004). In the endosperm tissue, the storage materials were accumulated via different ways, for example, from the marginal part to the center (such as in *Z. fabago*), and from the micropyle pole to the chalazal region. In wheat, the starch biosynthesis initially occurs in the cells of the central part and finally, the accumulation of starch grains in these cells is higher than marginal cells (Xu et al., 2011). During the embryo development in *Z. fabago*, the reduction of starch grains in endosperm was not recognizable; therefore, the starch grains may possibly have low contribution in embryo nutrition.

On the other hand, with gradual hydrolysis of starch grains simultaneous to the thickening of the thin cell walls of the endosperm, starch grains seem to be used for thickening of cell walls. Thick cell walls of the endosperm may remain even after maturity. Therefore, the cell walls could possibly be hydrolyzed after seed germination and could be used by young seedlings. The mentioned results are in accordance with the findings obtained by Figueiredo and Kohler (2016).

The ontogeny of the endosperm is associated with the seed coat development. Actually, the initial signals of the seed coat establishment are produced by the fertile central cells/endosperm (Weijers and Wagner, 2016). In addition, through activation and inhibition of genes, they contribute to controlling the signals for the seed coat development (Liao et al., 2015). Previous studies have suggested that the ovule integuments receive some signals (such as auxins) from the endosperm, causing the thickening of their cell wall (Creff et al., 2015). The thickening process of the seed coat cell walls in *Z. fabago* could be justified by this concept.

5. Conclusion

Our findings revealed that the anatropous ovule of *Z. fabago* is bitegmic. During the seed development, numerous and interwoven strings were formed in the exotestal cells. The strings contained both polysaccharides and polyphenols in their structure. In the late stages of the seed development, the nucleus and cytoplasm of exotestal cells were declined and the sculptures were evident on the seed coat. In seeds of *Z. fabago* development of endosperm occurred very fast, and embryogenesis began later. From the globular embryo stage, the only important changes in the endosperm were the formation of starch grains and an increase in their volume. Starch grains seemed to be used later by the developing embryo. In contrast, in the exotestal cells, only increase in the cell volume and depletion of starch grains came to pass. During the late embryogenesis stage, thickening the cell walls and accumulation of lipids and proteins in the cells of endosperm were observed. Actually, the cell walls in endosperm tissue seemed to be thickened to protect the embryo and to save carbohydrates.

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