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Histological study on the stages of pollination and fertilization in the cultivars of red seedless and ghezel-ozum grapes

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ABSTRACT

The Vitaceae is one of the most important plant families,which it includes the *Vitis* genus with all its economic characteristics. In the meantime, the European seedless grapes for their high-quality fruitshave popularity. However, in the breeding works, the progenies from the seedless cultivars have a low frequency. In Sultana cultivar (known as Thompson seedless in the United States) which is the most important seedless cultivar, abortion of the embryo after pollination and fertilization has been mentioned as a reason for being seedless. Investigating the structure of the seed and determining the time of abortion can be important in terms of tissue culture, embryo rescue and biology. In this research, the comparative study of the seed development of the red seedless grape with Ghezel-Ozum seeded cultivars considered using the histological techniques. Samples were harvested from pre-pollination till seed maturation every five days from the Research Station of Kahriz, Urmia and fixed in the FAA fixator. The fixed samples were immersed in the paraffin and then were cut with a microtome. After staining with PAS-Hematoxilen, samples were studied with the light microscope and photography. Observations showed that the growth and development of zygote and endosperm were delayed and eventually stopped in the red seedless cultivar after the double fertilization.

KEY WORDS: ABORTION; DOUBLE FERTILIZATION; ENDOSPERM; POLLINATION; SEEDLESS GRAPES; SEEDED GRAPES

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INTRODUCTION

The Vitaceae is one of the most important plant families, which it includes the *Vitis* genus with all its economic characteristics. Furthermore, this family has about 15 genera with 900 species (Zhang *et al.* 2015). The *Vitis* genus has a special importance in horticulture. Itis divided into two sub-genera of Euvitis and Muscadine. The commercial cultivars are related to the first sub-genus. Their flowers have 5 sepals, 5 petals, 5 stamens linked to the petals and alternating with the disc-shaped edges and the pistil with di-carpel ovary, in which each ovary there are the two standing ovules (Ghahraman 1993; Ghanadha *et al.* 2004).

The different species of the sub-genus Euvitis (2n=38) have very little differencesin terms of the chromosomal structure, and their intercourse with each other is possible. Therefore, only the geographical boundaries and ecological barriers have separated them from each other and created the European, American and Asian varieties (Ghahraman 1993; Ghanadha *et al.* 2004).

The European grapes due to having high-quality fruit as the commercial varieties are popular, and it has been found that polyphenolic compounds existing in those which include the flavonoid compounds are useful in preventing the heart diseases and cancer in human (Kalt 2001; Lepiniec *et al.* 2006). The American grapes are also used as the stock due to resisting pests and diseases. The varieties that are resistant to cold and frost can also be found in the Asian grapes (Ghahraman 1993; Ghanadha *et al.* 2004).

The origin of the European grapes is the area between the Caspian Sea to the Black Sea and from there to the Mediterranean area and Europe has expanded (Wen 2007). Although, grapevines are the dioecious plants, but following selective breeding done by man that has continued for many years, the male stocks have been removed from the European grapes and now the female stocks and self-pollinated hermaphrodite are cultivated. Since being seedless is desirable characteristic in many fruits for table utilizations, in the grape has also paid special attention to this feature. However, the seedless varieties and their progenies have a low frequency. Perhaps the most main factor is difficulties in making hybrid and producing seed in these varieties (Bharathy *et al.* 2005; Liu *et al.* 2003; Ramming *et al.* 1991; Sharma *et al.* 1996; Yang *et al.* 2007).

In Sultana cultivar which is the most important seedless cultivar, being seedless is the result of digesting the embryo after pollination and fertilization. Ebadi *et al.* (2010) demonstrated that the high frequency of abnormal ovules and single fertilization can be considered as two other reasons of seedlessness. The remainders of undeveloped ovule inside fruitcan be seen until its harvest stage. However, due to the small size of these immature seeds, it does not feel when eating fruit. Although this feature is very favorable in terms of production, but in terms of eugenic objectivesand obtaining the reproductive progenies would be problematic (Bharathy *et al.* 2005; Farsi &tBagheri 2004; Farsi &tZolAli 2006; Liu *et al.* 2003; Wakana *et al.* 2002; Yang *et al.* 2007).

Knowing how to digest the ovule and its morphological disorders and the appearance time of this phenomenon is very important and necessary. Perhaps only by this way, the separating of embryo and timely rescue it can be performed (Bharathy *et al.* 2005; Liu *et al.* 2003; Yang *et al.* 2007).

Therefore, the comparative study of the development of the ovule, the formation of embryo sac and seed in the seedless and seeded cultivars of grapes not only explain the structural and morphological differences between them, but it can also use to identify and utilize the appropriate methods to prevent the hollowness appearance of the seed (in breeding purposes) (Pratt 1971).

More processes of the development of ovule and the appearance of embryo occur at the microscopic level thereforethe use of appropriate histological methods for the structural studying of seedless in the grapes will be inevitable. However, preparing the microscopic samples of the perennial trees has specific problems which the hardness of tissuesas well as tannins and other phenolic compounds in them can be noted, and their combination with chemical materials used in the fixator prevents the optimal fixation of the tissues. Therefore, cellular and histological studies on tree species much less than herbaceous plants, and vines are not also an exception. In the annual and biennial plants such problems are less common (Ruzin 1999).

The purpose of this study was a documentation of histological information in *vitis* geneus with a comparative study of the seed development of the red seedless grape with Ghezel-Ozum seeded cultivar to exact determine the time of embryo abortion. This study can be useful in studies and practices of tissue culture and embryo rescue. Because, to know an appropriate time of exiting seeds can be more successful in tissue culture and embryo rescue.

MATERIALS AND METHODS

PLANT MATERIAL AND SAMPLING

Samples (flower buds, flowers and fruits during sampling) were collected from Seed and Plant Improvement Research Institute, Kahriz, Urmia, Iranin Jun to August 2007. Samples were collected from 10 days before and 40 days after the loss of cap (with a 7-day intervals) (one inflorescence per branch, and 10 branches per plant and 20 plants in the total of the experiment for each

variety), and immediately fixed in the FAA fixator (formaldehyde- acetic acid-alcohol) and transferred to the laboratory. The local varieties that were used for experiments, including the red seedless cultivar (containing stenospermocarp seed) and Ghezel-Ozum seeded cultivar (containing the actual seed) were selected for comparison with each other.

Samples were harvested from the clusters that were in the similar phonological stage, and were marked for next sampling. Samples were harvested in the early morning to avoid shrinking and losing the water. The 8-year plants were used and cultivated by using the method of Top Wire Cordon (2×4 Meter).

Samples were kept in the refrigerator during the experiment period. Then, based on the length (in mm) of pistil (or ovarian), and fruit were dividedin the early and later stages, respectively. These divisions in the early stages with the graph paper, and at later stages with the caliper under the simple (loop) microscope were done In the maturity stages, seeds were separated from the fruits and their length were also measured.

Separating under the loop was done using forceps and sharp-pointed needles, and cap and stamens were precisely separated, and immediately placed in the fixator materials. Seeds were also separated from the fruit in the same method. At this stage, it has paid attention to the time of separating not to damage the samples. Furthermore, in the mature seed, the embryo was removed from the seed and examined under a microscope. Sampling of the Ghezel-Ozum and red seedless cultivars were performed in accordance with Tables 1 and 2.

PREPARING THE SAMPLES AND HISTOLOGICAL ASSAY

The samples were immersed in the FAA fixator (formaldehyde 37%, 5 ml; ethanol 50%, 90 ml; and 5 ml of glacial acetic acid) for 12-24 hrs. After sufficient washing with running water and dehydrating with increasing levels of ethanol, the samples were clarified with xylene and saturated with paraffin. The samples after molding in paraffin, with the rotary handle microtome (R Jung Heidelberg) were cut at the thickness of 8 to 10 µm. The slices after removing paraffin and water were stained with Hematoxylin and PAS-Hematoxylin (Jenson 1962). The staining of Light Green, and Sudan Black and Red were used for studying proteins and lipids, respectively (Gahan 1984). The microscopic investigatingand photographing of samples were done with the light microscope (Nickon, E200-LED, USA).

Table 1. The dates of sampling of the Ghezel-Ozum cultivar

Date	Developmentstage	Features
2008/05/28	The stage of pollination	The peak stage of anthesis The length of ovule: 1-1.5 mm
2008/06/02	5 days after pollination	The length of ovule: 2-3 mm The length of fruit: 4-6 mm
2008/06/07	10 days after pollination	The length of seed: 2-4 mm The length of fruit: 8-10 mm
2008/06/12	15 days after pollination	The length of seed: 5-6 mm The length of fruit: 10-14 mm
2008/06/17	20 days after pollination	The length of seed: 6-7 mm The length of fruit: 14-16 mm
2008/06/24	27 days after pollination	The length of seed: 6-8 mm The length of fruit: 14-18 mm
2008/06/27	30 days after pollination	The length of seed: 6-8 mm The length of fruit: 18-20 mm
2008/07/03	36 days after pollination	The length of seed: 6-8 mm The length of fruit: 18-20 mm
2008/07/07	40 days after pollination	The length of seed: 6-8 mm The length of fruit: 18-20 mm
2008/07/13	46 days after pollination	The length of seed: 6-8 mm The length of fruit: 18-20 mm
2008/07/18	51 days after pollination	The length of seed: 6-8 mm The length of fruit: 18-20 mm
2008/10/30	5 months after pollination	The length of seed: 6-8 mm The length of fruit: 18-22 mm The stage offull maturity of embryo and a ripe fruit

Table 2. The dates of sampling of the red seedless cultivar

Date	Developmentstage	Features
2008/06/13		Flower bud
2008/06/14		The beginning of anthesis
2008/06/16		30 to 50 percent anthesis
2008/06/18	The time of pollination	The peak stage of anthesis (80 percent flowers in anthesis) The length of ovule: 1-1.2mm
2008/06/21	5 days after pollination	The length of seed: 1.2-1.5 mm The length of fruit: 2-4 mm
2008/06/27	10 days after pollination	The length of seed: 1.5-2 mm The length of fruit: 4-6 mm
2008/07/03	15 days after pollination	The length of seed: 2-2.5 mm The length of fruit: 6-10 mm
2008/07/08	20 days after pollination	The length of seed: 2.5-3 mm The length of fruit: 10-10 mm
2008/07/15	30 days after pollination	The length of seed: 2.5 mm The length of fruit: 12-14 mm
2008/07/28	40 days after pollination	The length of seed: 2-2.3 mm The length of fruit: 12-14 mm

RESULTS

THE CHANGES OF PRE- AND POST-POLLINATION STAGES IN THE RED SEEDLESS CULTIVAR

In the flowers of Red Seedless cultivar in the anthesis (stage 4; stages 1-3 are not shown here) based on sampling date and the morphological studies with the light microscopy, the pollen shad germinated and pollination had occurred. It should be noted that the flowers in grapes are on the clustered inflorescences and at one inflorescence, flowers can be in several different phonological stages, and in examinations should be considered this issue.

Three days after pollination (stage 5), the fertilization occurred in all the flowers, and micropyle was been closed, which was due to growing upand becoming massivethe internal walls.At this stage, the presence of pollen tube into the embryo sac was significant (Fig.1).

The microscopic sections prepared from flowers pollinated at the stages of 10 and 15 days after pollination showed that the double fertilization in this cultivar was successfully occurred; so that the first division of the zygote was observed in 15 days after pollination. The apical and basal cells were formed in high and low densities, respectively and the basal cells were observed in its first division (Fig.2).

In the stage of 8 (20 days after pollination), a number of the free nuclei within the embryo sac were observed which have been resulted from the division of synergids (Fig.2). Whereas at this stage the growth of the zygote had been stopped and the cell had been degenerating. The formation of the brown sediments around the cell that was degenerating was significant (Fig.3). The significant point at this stage was distinctively the thickening of the transverse walls of calot cells which had likely been started from the previous stage (the tangential walls). Furthermore, sometimes the wall thickening was observed in the radial walls (Fig.3).

In addition, other notable phenomenon was the separation of the inner integumentfrom the outer integument which was started from this stage and at the following stages, this space was increased (Fig.4).

In the stage of 9 (30 days after pollination) in the red seedless cultivar, the nucellus were strongly pressed and the inner integument completely kept away from the outer integumentand an empty space was created between them (Fig. 4b).

In the stage of 10 (40 days after pollination) in the red seedless cultivar, the shrinkage of nucellus was continued and nucellus in the form of crumpled on one side of the seed was observed. The stages of degeneration were along with the shrinkage and becoming small of the seed. Thickening in the transverse walls of the calot cells were also continued at this stage. In the outer integument, the needle-shaped crystals of calcium oxalate were observed which large groups of them were formed within the idioblast (Fig. 5).

THE CHANGES OF PRE- AND POST- POLLINATION STAGES IN THE GHEZEL-OZUM CULTIVAR

Five days after pollination in the Ghezel-Ozum cultivar, zygoteand synergids were observed in the embryo sac (Fig.6).

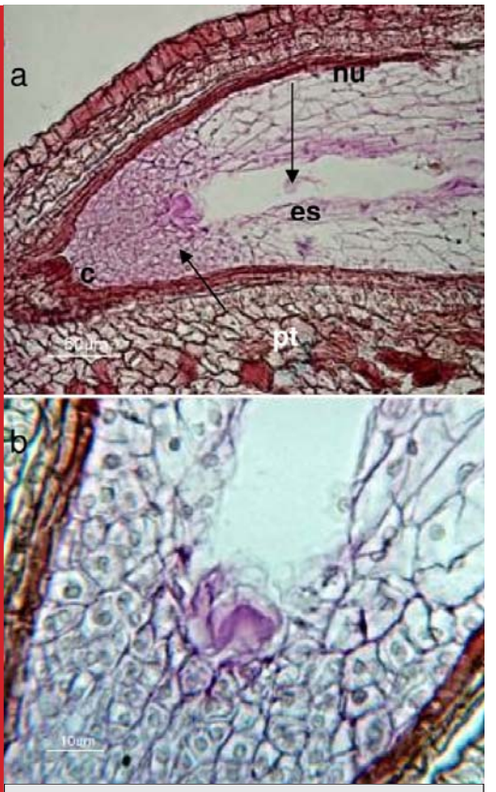

FIGURE 1. The longitudinal section of the seed of the red seedless cultivar in 3-5 days after pollination (stage 5), stained with the PAS-Hematoxilen method, a) Ovule-synergids and polar nuclei within the embryo sac were distinguished. b) Fig.¬a with further magnification, the entering of the pollen tube to synergids was distinguished. c: calot, es: embryo sac, nu: nucellus, pt: pollen tube.

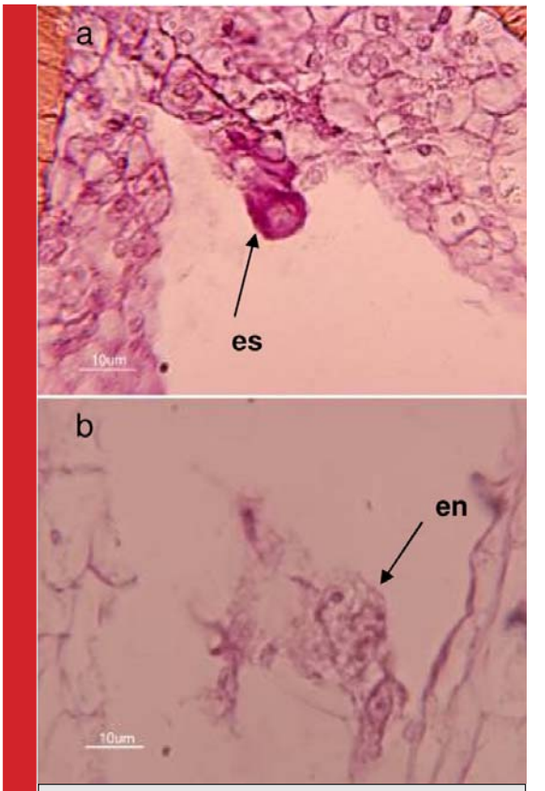

FIGURE 2. To occur the double fertilization process in the red seedless cultivar, stained with the PAS-Hematoxilen method. a) The zygote has fertilized and its first division has done. b) Endosperm nuclei within the embryo sac are identified. en: endosperm nuclei, es: embryo sac

Ten days after pollination, a number of the free nuclei were observed within the embryo sac. The free nuclei, after the double fertilization, were resulted from the divisions of the synergids which would form the endosperm. The zygote as well as the synergids was observed in the form of the shrunk mass by the side of that (Fig.7). In the outer epidermis of the inner integument, the tangential divisions were seen with the increasing of the lengthin the radial direction. In the inner epidermis of the outer integument were also added on the reddish brown compounds.

In Ghezel-Ozum cultivar, the formation of the globular embryo was observed in simultaneous with the stage of 8 (20 days after pollination) (Fig.8). In addition, in the Ghezel-Ozum cultivar, endosperm was formed with cell wall and also both internal and external integuments grew.

In the final stages, the entire seed with the mature embryo and the cell endosperm was observed. The embryo in this cultivar had thoroughly evolved and consisted of an axial section, which the apical meristems of the root and stem formed at the two ends of that, and storages-accumulated cotyledons were on the both sides of the apical meristem of stem (Fig.9).

In the mature stage of the Ghezel-Ozumseed, the protein compounds within the endosperm tissue were thoroughly observed which were stained with Light Green (Fig.10). Furthermore, the needle-shaped crystals of calcium oxalate in the form of the groups of idioblast were also observed in the final stages in this cultivar.

DISCUSSION

One of the important cultivars of grapes is red Sultana or red seedless which is one variety of stenospermocarps and consumed freshly and for producing sultana. So far, few studies, histologically, was performed on this cultivar. Stott (1936) was the first to report stenospermocarpy and he applied this word for the immature seeds. He

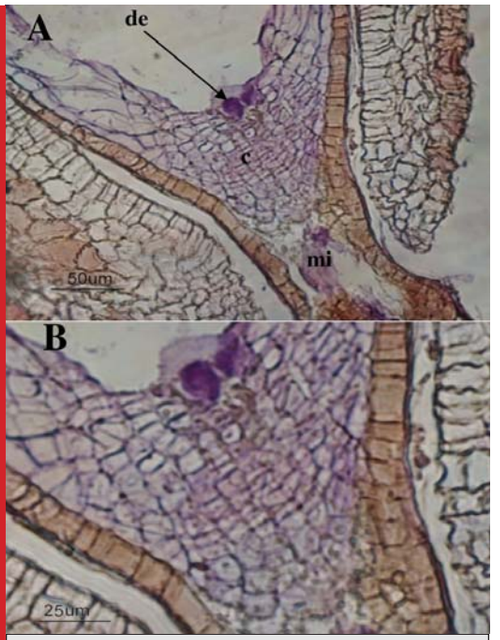

FIGURE 3. Longitudinal section of red seedless cultivar in 20 days after pollination, stained with the method of Hematoxilen .a) The degenerated pre-embryonic cells were shown at the micropylar pole. B) Fig.a. With further magnification. Brown sediments are observed around the purple mass. c: calot, de: degenerated embryos, mi: micropyle

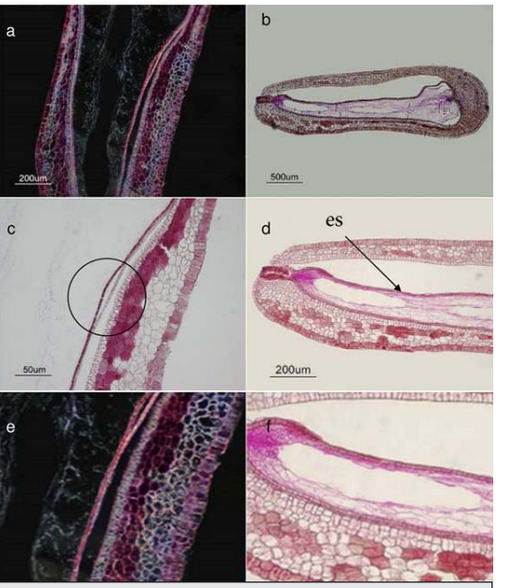

FIGURE 4. The longitudinal section of red seedless cultivar in 20-30 days after pollination (stages 8 and 9), stained with the method of PAS-Hematoxilen. a) The initiation of separating the inner integument from the outer integument is shown. Photographed with dark field microscope, 20 days after pollination (stage 8). b) Separating the inner integument from the outer integument, 30 days after pollination (stage 9). c,e) Fig. a with further magnification.d, f) Fig. b with further magnification.The nucellus has degenerated. es: embryo sac.

stated that the growth of embryo in Thompson Seedless varieties continues to globular embryo stage (according to the Pommer *et al.* 1995).Thereafter, it has been more considered the breeding aspects of grapevines, which the most important subjectswere embryo rescue with the aim of disease resistance, improving fruit quality and enhancing the performance in the stenospermocarp cultivars. In these studies, genotype, culture medium, sample age and harvest time have been introduced as important factors for successin embryo rescue, however, any exact research has not been performed on the subject of ovule and immature seeds of stenospermocarp (Sharma 1996; Yang *et al.* 2007).

Studies showed that pollination at the stenospermocarp cultivars is just a factor to develop fruit, and following growth and development of endosperm and the embryo have difficult (Hanania *et al.* 2007; Pratt 1971). Pratt (1971) noted that the outer integument of ovule at the stenospermocarp cultivars is without sclerenchyma cells and ovules or aborted seeds remain just as a small object into cubes. The development of ovule may be normal, such as seeded cultivars that have nucellus, one or two integuments and the cord, and or is nearly normal. Pratt (1971) noted that pollen is usually fertile and self-pollination occurs in these cultivars. It was found that the being seedless characteristic is heritable (Bouquet and Danglot 1996; Pratt 1971). Liu *et al.* (2003) demonstrated that the main reason of stenospermocarp in Sultana seedless grapeis unknownup to now.

GERMINATION OF POLLEN

It was found that pollen affects the growth and size of seed, because, the half of the embryo genes and one third of endosperm genes are sourced from paternal parent (Ebadi & Dehghani 2002). Pratt (1971) said that stenospermocarpc ultivars have usually alive pollen. In our surveys on the red seedless grape, viability and germination of pollen on the stigma surface were confirmed by fluorescence microscope, however, because of style tissue thickness, the observation of its penetration from stigma surface to style was impossible. In sectioning to view in bright field light microscope, the pollen tube and its transition place in the micropylar region and calot were observed which shows the pollen tube penetrates into the ovule and then embryo sac.

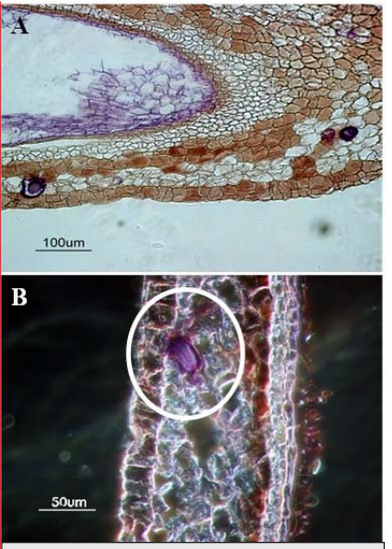

FIGURE 5. The longitudinal section of red seedless cultivar in 40 days after pollination (stage 10), Stained with the method of Hematoxilen. a,b) Parenchymal cells containing a mass of calcium oxalate crystals (Idioblast).

FERTILIZATION AND ZYGOTE FORMATION

In studies, it has been found that the pollen tube reaches the micropyle within 12 hrs and fertilization take place 24 hrs after pollination (Pratt, 1971). However, some literatures demonstrated that it occurs 2 to 3 days after pollination (Batigiana 2006). In an effort to breed seedless varieties by Sahijram and Kanamadi (2003), oocytes were histologically investigated 24 hrs after pollination, and the formation of zygotes were observed in 4 days after pollination at all studied crosses. Endosperm mother cell was also formed in all of them, and it means that double fertilization was successful (Sahijram & Kanamadi 2003). These findings correspond with our observations. The studying sample appeared that fertilization occurred in 3 to 5 days after pollination (due to a clustering of inflorescence and intervals for sampling). In the studied microscopy sections at this stage, the presence of pollen tubes inside the synergids confirms this. Also, fertilization at Ghezel-Ozum cultivar took place in 3 to 5 days after pollination.

POST-FERTILIZATION

Endosperm formation

In our surveys, after fertilization, free nuclei were formed from divisions of the synergids nucellus in the embryo sac. However, 30 to 40 days after pollination,

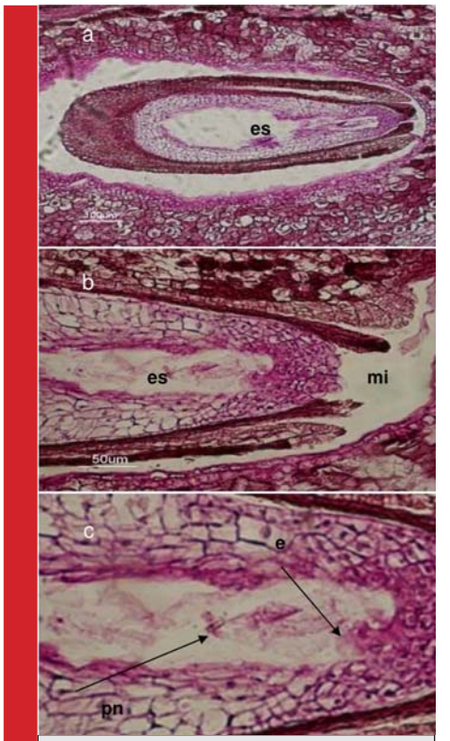

FIGURE 6. The longitudinal section of Ghezal-Ozum seedless cultivarin pollination and fertilization stage (stage 5), stained with the method of PAS-Hematoxilen. a) Longitudinal section of ovule at the Ghezel-Ozum cultivar. b) Embryo sac in the micropylar pole with further magnification has been determined. c) Zygote cells and ovule nuclei have been identified. e: embryo, es: embryo sac, pn: polar nuclei, mi: micropyle

it was found that the endosperm cells were gradually degenerated at this stage; a type of shrinkage was also created in nuclei.

In some literatures were noted that the growth of embryo can be stopped and aborted at the immature stage which are mentioned different reasons for this. For example, it can refer to the lack of proper nutrition of embryo by the endosperm (Bharathy et al., 2005; Liu et al., 2003; Ramming et al., 1991; Sharma et al.,1996; Yang et al., 2007). It was also distinguished that the death of the embryo can be due to toxin production by endosperm and incompatibility of embryo and endosperm (Bharathy *et al.* 2005; Yang *et al.* 2007). In red seedless cultivar, it seems that one of the reasons for stopping growth and development of zygote is the stopping the growth and development of the endosperm; however also the delay in the growth and development can be another

FIGURE 7. The longitudinal section of the seed of Ghezel-Ozum cultivar in the 10 days after pollination (stage 6), stained with the method of PAS-Hematoxilen. a) Zygote and primary endosperm nuclei are shown at the micropylar pole. B) Fig.a.with further magnification, zygote and endosperm nuclei which are dividing have been identified. c) Zygote with further magnification was shown. ic: inner integument (coat), mi: micropyle, ne: nuclear endosperm, z: zygote, c:calot

cause. Pachno *et al.* (2014) suggested that, in apomictic dandelions, the persistent synergids may play a role in the nutrition of the developing embryo.

In Ghezel-Ozum seeded cultivar, endosperm tissue is formed and fully developed after double fertilization. Ten days after pollination, the release endosperm nuclei were observed in large numbers, which their growth has continued in the following stages in 15 to 20 days after pollination and eventually was fully shaped into cell. In our investigations, proteins, polysaccharides, oil compounds and star-shaped crystals of calcium oxalate were observed in the mature endosperm.

Endosperm in the seeded cultivars of grape has irregular shape and is mainly composed of thick-wall cells (Pratt 1971). It was found that the endosperm can continue to grow even without fertilization (Chaudhury *et al.* 1998; Raghavan 2006). Endosperm effect on embryo morphogenesis is probably due to physical pressures and in the production of somatic embryos can be observed abnormal mode due to imbalances in pressure (Farsi &Bagheri 2004; Farsi&ZolAli2006).

GROWTH OF INTEGUMENT AND CLOSING THE MICROPYLE

Batigiana (2006) demonstrated that a series of severe divisions after fertilization occurs in the funicle, navel, chalaza and integuments.Also, in the seedless cultivar, after fertilization, the growth of cells of inner integument was observed and therefore micropyle was closed. Then any division was not observed at the integument cells, while in Ghezel-Ozum seeded cultivar, the growth of outer integument was impressive. Carraro *et al.* (1979) reported when micropyle remains open it is due to the disruption of pollination, and increases the embryo abortion and stenospermic in grapes. In our observations, micropyle opened before fertilization, and after fertilization due to the growth of inner integument cells had been closed. Haughnand Chaudhury (2005) reportedthat there is a relationship between the growth of endosperm and integument which is related to genetic control. It means that with growth and development of the endosperm, integument growth continues. Striem *et al.* (1992) showed that seed integument and endosperm formation and development in the stenospermocarp grapes are independent of each other, and therefore, seed integument will be emerging without endosperm and embryo. In our study, independent of the integument formation from endosperm and embryo was rejected; because in this sample, growing integument of embryo, i.e. the seed, was stopped from 20 days after pollination and even earlier when also stops growth of endosperm.

In our studies, it was found that the outer integument at micropylar pole was formed from two to three cell layers and in chalazal pole was added to the number of layers and it is 6-7 cell layers.In the red seedless cultivar, this integument has color compounds that are likely polyphenols or anthocyanins. Vessels at the chalazal pole of outer integument were completely visible.The Color of outer integument was different with inner integument and has transparent cells that contain clearcontents.The inner integument by two to three layers has the compressed, stretched and flattened cells astangential. The inner integument surface was covered by the cuticle layer which was determined after staining with black Sudan. These observations correspond with the findings of Pratt (1971).

In the following stages of the red seedless cultivar, we saw the separation of the inner integument from the outer integument. The beginning of this phenomenon was observed 20 days after pollination. Furthermore, nuclei were gradually suffering from degeneration and

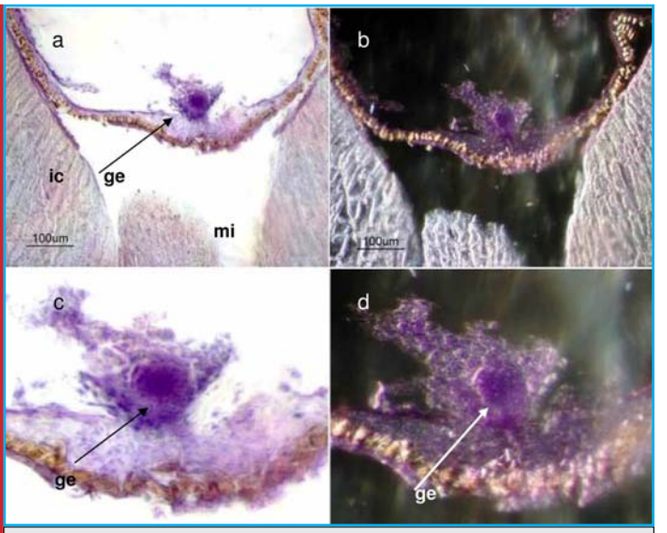

FIGURE 8. Axial sections of the seed of Ghezel-Ozum cultivar in 20 days after pollination (stage 8), stained with method of Hematoxilen. Photographed with dark and bright field microscope.a) Globular embryo is shown at the micropylar pole. Photographed with a bright field microscope.b) Globular embryo is shown at the micropylar pole. Photographed with a dark field microscope. c) Fig. a is shown with further magnification. d) Fig.b. Is shown with further magnification. ge: globular embryo, ic: inner integument (coat), mi: micropyle.

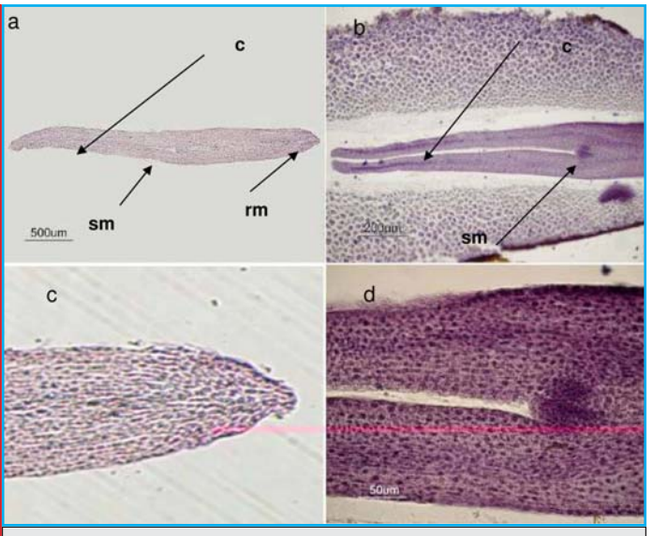

FIGURE 9. Longitudinal sections of mature embryo of the Ghezel-Ozum cultivar, stained with the method of Hematoxilen. a) Longitudinal section from isolated embryo, meristem of roots, shoots and cotyledons has been specified. b) Longitudinal section from embryo inside the seed, meristem of shoots and cotyledons has been specified. c) Root meristem. d) Shoot meristem. c: cotyledons, rm: root meristem, sm: shoot meristem.

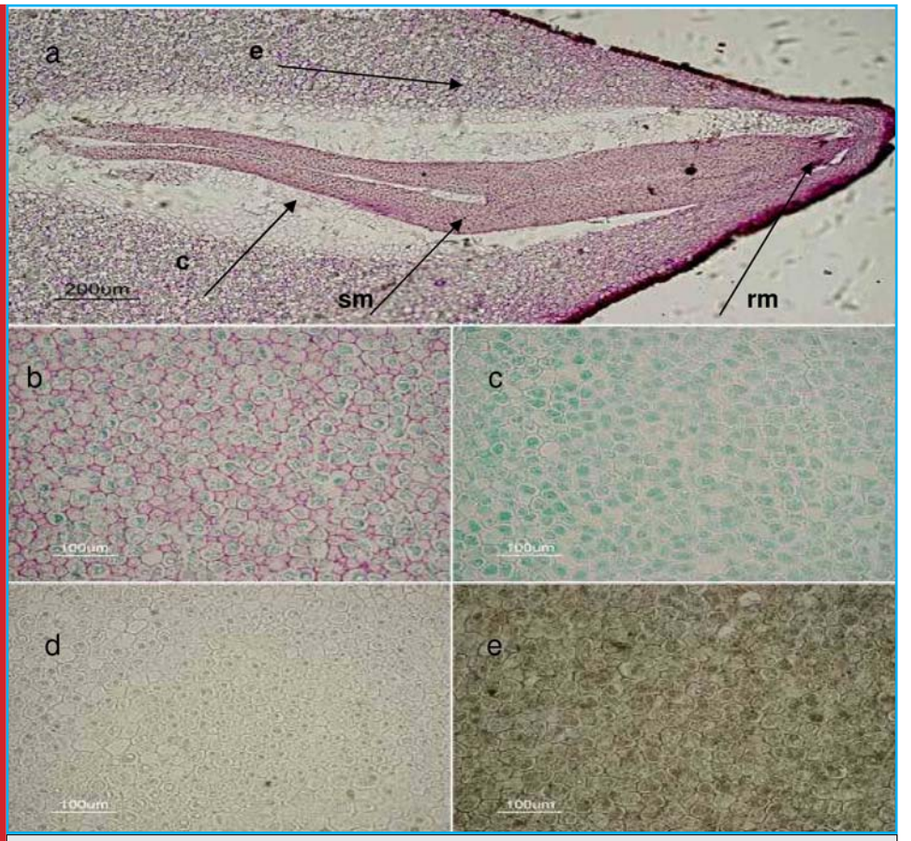

FIGURE 10. Longitudinal sections of mature seed of the Ghezel-Ozum cultivar, the different parts of the embryo and endosperm components are shown, stained with the method of PAS_Hematoxilen. a) Embryo is shown inside the endosperm tissue. b) Endosperm tissue that stained with the method of PAS- Light. In this figure, polysaccharide walls and intracellular storage are shown green.c) Endosperm tissue that stained with the method of Light green. In this figure, protein components are shown. d,e) Endosperm tissue that stained with the method of black and red Sudan, plasma membrane of cells with Sudan Black, is stained black. c: cotyledon, e: endosperm, rm: root meristem, sm: shoot meristem.

shrinkage. These findings are corresponded with studies of Vallania *et al.* (1987) in terms of shrinkage of nucleolus and separating integument.

FINAL STAGES

With further investigations it was found that the zygote divided 7 or 15 days after pollination. In fact, the zygote has done fist division and then stopped.The maximum size of seed is created at the end of the first stage and then its growth stops and just continuing the growth and development of the embryo, endosperm and berries would be observed. In our observations and investigations, also seed size was completed 20 days after pollination, but then, growth of embryo had stopped.

In the red seedless cultivar, following the investigations, on 20 to 30 days after pollination, brown sediments around the zygote which is dividing, were observed that seems be the compressed polyphenols and tannins and may be effective in the death of embryo.This compact tannin role as an agent for dormancy and preventing earlier germination of the seed was documented which are in the seed coat (Debeaujon *et al.* 2000). Furthermore, nucleolus had been suffering from shrinkage in this stage. At the final stages, needle-shaped crystals of calcium oxalate were observed at the outer wall that large groups of them formed within idioblast, which their roles are for regulation of calcium, plant protection, detoxification of heavy metals, ion balance, firmness and so on (Vincent *et al.* 2005).Also, Rosianski *et al.* (2016) reported that the pollinated fruit of fig had a larger diameter and weight and improved firmness compared to the parthenocarpic fruit. These groups of calcium oxalate were also observed in the seeded cultivar.

The calcium oxalate crystals were also observed within the seedin the final stages. Pratt (1971) noted that this case can be observed in samples with abnormal ovules that oxalate crystals are replaced by nucellar cells. These abnormal ovules consist inovules with nucleolus and embryo sac with defection at growth and development, abnormal curvature of ovule in the ovary, necrosis of part of chalaza and so on.

CONCLUSION

The findings showed that growth and development of zygote and endosperm were delayed and finally stopped in red seedless cultivar after double fertilization. It means that first zygote was divided in 15-20 days after pollination when apical and basal cells had been formed, but after ward their divisions were ceased. Also in 20 days after pollination the series of brown sediments were observed around the zygote. Mean while, in Ghezel-Ozum cultivar, the formation of globular embryo and endosperm were observed in 20 days after pollination. In red seedless cultivar, endosperm cell was divided as nuclear that release nuclei were observed in the embryo sac and then divisions were stopped. Moreover, in the transverse walls of calot cells, thickening was seen quite clear. At the nuclei in the final stages, shrinkage and degeneration were observed. Also, internal integument was separated from outer integument.

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