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ORIGINAL ARTICLE

Histological study of embryo formation in *Tribulus terrestris* from harmal spin

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Embryo development occurs continuously in plants, but for better description, the following steps are considered: Pre-embryonic stage, spherical stage, cardiac stage, spinal-shaped stage, and eventually adult embryo. The study of embryonic development and development of endosperm in various plant genes is very important for phylogenetic and systematic studies. The purpose of the present study is to investigate the histological evolution of fetal development in Tribulus terrestris from Harmal spin using optical light microscopy methods. The ovaries and fruits of this plant were collected from nature and fixed in the FAA fixer. In the next step, the fixed samples were studied by different Histological and the Histochemical methods. According to histological and Histomorphological studies, the symmetric Dicotyledon of the egg cell led to the formation of an apical and basal cell. Divisions occurred in the basal cell faster than the apical cell and a filamentous suppository was produced. The embryo was formed as a result of divisions in the apical cell. The suspensor disappeared in the throat embryo stage. Mature embryo was 3.5 mm long. Based on histological studies, endosperm was a nuclear material that had no storage material during embryonic development. With the growth of the embryo of the endosperm cells and their cell walls became close. In the mature embryo stage, endosperm was found in the shape of a tubular structure around the section and between us in it.

Keywords: Endosperm; *Tribulus terrestris* from Harmal spin; chemical hysteresis; FAA fixer

Introduction

Understanding the nature and mechanism of the processes that lead to the formation of the existing plant is an important issue of biology and it is not possible to spread this science without knowing the early stages of ontogeny (Batygina, 2006). Embryo plays a key role in the life cycle of flowering plants. This process is divided into three plants:

- First stage: is the morphogenesis stage. At this stage, the embryonic axis and Dicotyledon are formed.
- Second stage: The embryo's stage of puberty is associated with the storage of macromolecules, including storage proteins, lipids and carbohydrates in all embryonic cells, especially in Dicotyledon.
- Third stage: It is a stage in which the fetus develops the ability to dry and enter a period of metabolic resuscitation and enter the sleep or dormancy phase (West and Harada, 1993).

The most important events of this period are:

- Creating symmetry and cellular organization and forming apical root and stem,
- Transmission from the maternal nutritional heterotrophic state, to the embryo autotrophogenesis able to survive in the course of dormancy,
- seedling production after germination.

The development of embryos in flowering plants has several important features that distinguish it from the development of embryos in the animal system. These features include:

- The process of double fertilization followed by an interaction between the embryo and the endosperm tissue,
- Small size of the egg cell and its placement at the depth of the native tissue,
- Lack of cell migration during early stages of embryogenesis,
- The activity of a large number of zygotic genes during the early stages of embryogenesis,
- Formation of specialized cells to a small number,
- Formation apical meristems that produce mature plants.

The egg cell is the result of a double fertilization process. In many species, the egg cell is not immediately distributed after fertilization and has long or short-lived rest periods. During this period, significant intracellular changes occur in the egg cell. The polarity phenomenon in the egg cell is more pronounced, such that the dorsal scalar polarity and the vacuoles of the microphile polypeptide are fully recognizable. The duration of this period varies from plant to plant. For example in cotton is 60 hours and in Arabidopsis 30 hours after fertilization (Lersten, 2004; Sornsathapornkul and Owens, 1999). In addition, the egg cell during this period may remain constant in size, reduce in volume or grow in size and can be seen as stretched. For example, the size of the egg cell stays constant in the Hibiscus, reaches half the size of Brasicaceae in the Alcea, and grows and stretches in the darkened Brassicaceae of the egg cell before division and becomes visible as a single cell line (Lersten, 2004). The Tribulus terrestris species has Sinonium Tribulus panchinosus and is known as Kharkhasak in Iran, Pontovirin and Landcaltrop in the United Kingdom, and Negvalo and Sennagaloo in Canada and other names in other countries. A one year old herb is a broad-leaved grass on the surface of the soil. Young leaves and shoots are covered with elegant silk tufts. Leaves are a mixed pair of shoulder and often mutually interspaced 3-6 pairs on both sides of the main Petiole. The single flowers have 5 tufts, 5 yellow petals and 10 flags placed in two rings. The flags alternating with the petals are at the base of their nectaire. The ovary has 5 homes, simple cream and 5 branches. The fruit is in the form of a chisou-tapra (chistocarpus), which in time of arrival, separates and disperses the Meriacarp. Each of the outer surfaces of the Meriacarp has 2 sharp thorns in the apical and 2 hard spikes in the lower part. The species of Meriacarp live in the soil for many years and their dispersion is carried out by wool

or animal hair, clothing or shoes. The flowering season of this plant is from early June to late September. The best soil for growing this plant is dry and sandy soils, but this plant has a high tolerance to adapt to different types of soils (Figure 1) (Akhyani, 2000 & Fukuda, 1982).

Figure 1: Flowers and fruits of the Tribulus terrestris

Important events in the early stages of embryonic development in Tribulus terrestris are the arrival of egg cells in the resting period. This period lasts from the flowering stage (2.5 to 2.5 long) to the stage of the 3-mm length of the Meriacarps. During this period, the egg cell is enlarged and expanded and grown in 3-mm long in Meriacarp which can be seen. In addition, the polarity in the egg cell increases dramatically. After the rest period, the first division of the egg cell occurs and the egg cell is divided into two cells by horizontal wall-mounting. This division leads to the production of cellular cells and apical. In this research, attempts have been made to investigate the stages of development of embryo and endosperm in Tribulus terrestris in natural conditions and in maternal tissue. According to the mentioned stuff, the objectives of this research are:

- Histological and Cytochemicals embryo development by optical microscope,
- Histological and Histochemical changes of endosperm during embryo development.

Review of literature

Ragavan concluded in 1986, through the review of the results of a review of the substrate, in the Phaseolus genus and the apical family:

- In many suspensors, all cells or some cells have grown walls,
- In the suspensors, the synthesis of RNA and protein is superior to the fetus,
- There is evidence that suspensors cells have a high density of growth hormones than fetal cells.

Also, Ragavan defined the function for the suspensors:

- Absorption and active nutrient transfer,
- Fetal production in artificial conditions,
- The production of growth hormones for the regulation of early embryonic development.

The cell located between the suppository and the embryo is a pituitary in the 5-cell suspensor stage. This cell acts as a link between the embryo and the suspensors. The time, organization, and features of pituitary cell divisions are evident in various species. In 1934, Saux identified six types of pituitary:

- The pituitary cell is isolated, leading to the onset of cortex and root cap,
- The pituitary cell individually forms the middle part of the root cap. For example, in tobacco and cultivated poppy,
- Two-cellular Pituitary cells: 2 cells from one layer, or from two different layers of the pre-embryo, leading to the onset of cortex and root cap. For example, in Senecio vulgaris and nettle ball,
- A group of pre-embryonic cells differentiates into the pituitary tissue and provides cortex and cap:
    - Cells derived from the basal cell of a double-fetal embryo,
    - Cells derived from the double-cell embryonic apical cell.

In 1975, Swami concludes that the pituitary cell and its derivatives have a smooth root zone function. The pituitary cell and its derivatives are recognizable as small size, structural properties, and small amounts of protein from other pre-embryonic cells (Batygina, 2006). The results of this study indicate that the spherical phase of the pituitary cell is divided into longitudinal wall. Divisions that lead to the formation of embryos in the Angiosperms are done according to a very regular pattern in a large number of plants. Arabidopsis has been used for many years as a model plant for studying the process of fetal dividing in Dicotyledon. In Arabidopsis, two longitudinal vertical divisions of the ring cell cause the production of 4-cell embryo (Liee et al, 2006).

Sample and statistical population

Plant specimens including ovaries and fruits of Tribulus terrestris were collected from different stages of development from Tabriz in 2008 and 2009. For developmental studies, grouping of samples from the initial stage to the final stage is essential. In this research, in the first stage, closed buds, Fluffy flowers and dried flowers were cut. Then, the basis of the grouping was to increase the length of the Meriacarp and on this basis, eleven groups were identified:

- Step 1: Closed buds
- Step 2: fluffy flowers
- Step 3: dried flowers
- Step 4: Meriacarps with a length of 2.5 mm

- Stage 5: Meriacarps with a length of 3 mm
- Stage 6: Meriacarps with a length of 3.5 mm
- Step 7: Meriacarps with a length of 4 mm
- Step 8: Meriacarps with a length of 4.5 mm
- Step 9: Meriacarps with a length of 5 mm
- Step 10: Meriacarps with a lengh of 5.5 mm
- Step 11: Meriacarps with a length of 6 mm
- Step 12: Meriacarps with a length of 6.5 mm
- Step 13: Meriacarps with a length of 7 mm
- Step 14: Meriacarps with length of 7.5 mm.

At all these stages the Mericarps were green. From the stage of Meriacarps with a length of 7.5 mm, the cutting of them was not possible due to the lack of increasing the length of them and the ligninization of the radial walls of the internal epidermis cells of the Meriacarps. Therefore, from the stage of Meriacarp with a length of 7.5 mm later, the basis of the grouping was increased in grain length. According to this three groups were identifies:

- Step 15: grains with a length of 3 mm
- Step 16: grains with a length of 3.25 mm
- Step 17: grains with a length of 3.5 mm.

Method of the research

The excess thickness of the tissues and organs of the living organism in the natural state prevents the passage of light, thus the preparation of thin and transparent sections is essential for histological and cytochemical studies. The most commonly used method is to provide permanent slides of the tissues and examine them with light microscopy. Microtome was used for thin slices. Before cutting the slices, preparation of the samples was done as follows.

Fixing samples: The stabilizer was used for at least 24 hours. Of course, samples can be stored for a long time (several months) inside this stabilizer and at a low temperature (4 ° C). It should be noted that, due to the small size of the ovum and the softness of the ovary wall, the ovum and seeds were fixed within the Meriacarps, but in the rest of the stages, the seeds were directly stabilized by ligninization of the internal wall of the Meriacarps.

Molding: In order to mold, pure molten paraffin was poured into special molds and the specimen in the third paraffin bath was immediately transferred to the mold and placed on the mold floor in the desired direction so that the air bubble was not formed around the sample. Paraffin was quickly frozen when it was around the sample. To specify the specimen, the specification includes the name of the sample, the type of stabilizer and the date of molding in the label and placed next to the mold before the paraffin was frozen. In this way, the sample was arranged in a paraffin form and ready to be cut.

Cutting and fixing the slices on the slab: In order to cut the paraffin mold, it is necessary to modify it so that the sample is cut at the cutting surface and the trapezoidal shape (two parallel sides, one side with an angle of 90 and the other side with a 60 angle) is performed. Cutting was done by the R-Jung-Heidelberg microtome in a situation where the large trapezoidal side was placed down and parallel to the edge of the blade. Though the thickness of 7 micrometers is the most appropriate thickness for optical microscopic observations, then a 10-micrometer sample of embryonic sac was studied. In the next step, the paraffin bands obtained from the gelatin slab were fixed on a drop of water and on the Heater; therefore preparation of gelatin slimes prior to cross-sectioning was necessary.

Preparation of gelatinous slabs: The slabs were placed in a Sulfochromic solution for 12 hours. After this time, they were removed from the dissolved of Sulfochromic and washed under running water for several hours. Drying of the slabs is mandatory before placing them in a dissolved gelatin. Completely dried slabs were gently placed inside a flattened dissolved gelatin. After 20 minutes, they were removed from the solution and placed vertically on the support to allow the excess solution to flow and the slurry to dry. The slabs were dried in the oven at 40 ° C for 48 hours.

Coloring: Generally, most tissues are colorless and therefore it is difficult to observe them without coloring under a microscope. Different methods of coloring are invented that not only reveal different components of the texture, but also distinguish them from each other. This is done by applying a mixture of colors that selectively make the texture components more or less colorful. Most colors used in histological studies act like acid or alkaline compounds and form electrostatic bonds with tissue components. The slabs containing paraffin strips must be paraffin cleared and dispensed for coloring with specific color reagents. Paraffin dehumidification was performed by dipping the slabs in xylidol (fat solvent) and in order to accelerate de-paraffinization and the slabs and paraffin were heated for clarity before being placed in xylol on an electric heater. Subsequently, the slugs were placed in successive baths of ethanol in varying degrees. This step started with the addition of slabs in ethanol with higher degrees and ended with low grade ethanol. At the end of this stage, the slabs were ready to be painted. For histological studies, hematoxylin and the Hedoyceylene Predic acid-Schif method was used. In the hematoxylin method, the slabs were placed in the hemolysis solution after paraffin dehydration. Afterwards, the dehydration steps were complete and transported to xylol for clarification. Finally, using Antelan adhesive, Lamel was placed on the samples and permanent slabs were prepared. In addition, the pedik acid-shif hematoxylan slams containing stabilized sections in the FAA stabilizer were dispersed in aqueous pedik acid solution after the paraffin dehydration steps and were transferred to Reagent shale in distilled water. After washing with distilled water, they were placed in a hematoxylan solution, and then the dehydration and clarification steps were performed and the samples were colored with lamell and with a permanent adhesive Antelan.

Findings of the research

The structural study of the ovules by optical microscope showed that the ovules have two coats and have low elongation. The inner coating is composed of 2 layers of cells, which in the microfiber region increases the number of cell layers and it is surrounded by these cells. The outer coating on the funicular side has several layers of cuboid cells, the number of cell layers in the far side of the funicular Nousl reduced to 3 layers. Nousl has three layers of cells in the micro-fiber which is gradually increased to the number of cells in the direction of the Shalazi polar (Figure 2).

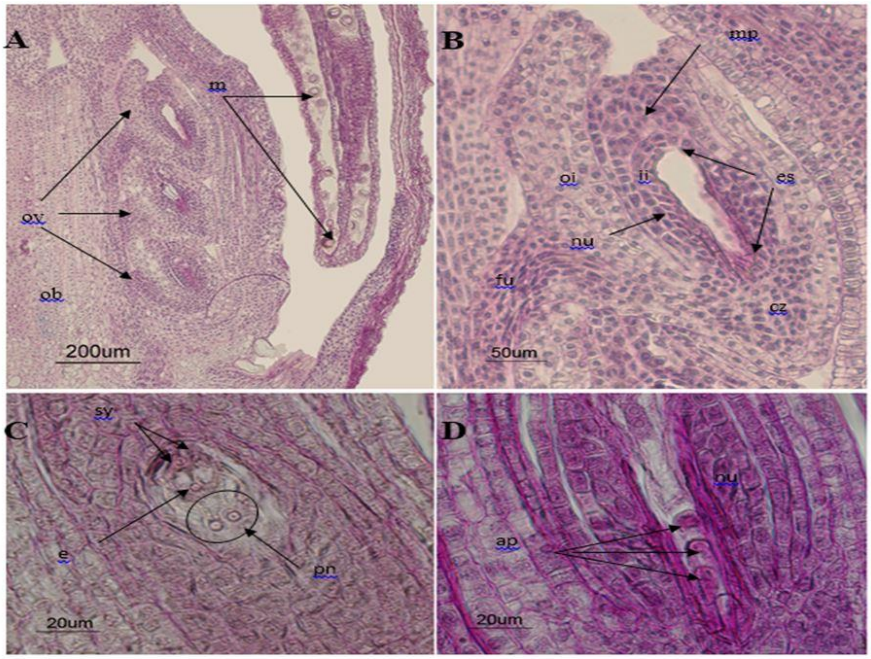

Figure 2: Longitudinal section of the ovary in the stage of closed buds by staining with hematoxylin-PAS (A: The structure of the ovary and how the oocytes are placed in each carpel. B: Shows the structure of the ovum. C: The structure of the embryo sac, which contains sedling cells, fibrils and synergites is shown. Ap: Anti-pedal, cz: Shalazi zone, e: seelding cell, es: Embryonic bag, fu: Bond, ii: Internal shell, m: Microspor, mp: Microfiber, ob: Ovary, oi: Outer shell, Ov: ovule, pn: polar nuclei, sy: synergy).

The results showed that, along with the maturation of the embryo bag and the fertilization process, the growth also increases and the number of cells and cell layers gradually increases in the Shalazi zone (Figures 3 and 4). In addition, with the growth of the texture of the nocturnal, the Noucl cells are separated from the inner lining, except in the Shalaz zone (Figures 4 and 5).

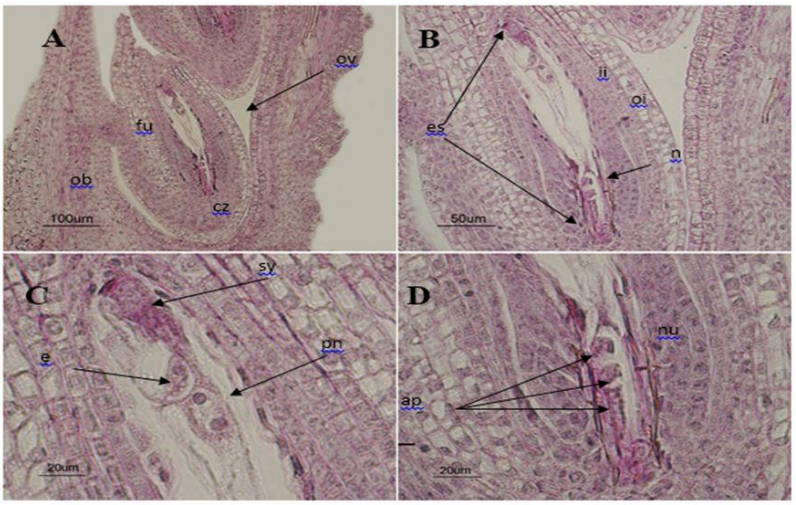

Figure 3: The longitudinal cut of juicy flowers is colored with hematoxylene-PAS: (A, B. The embryo's sac and ovum building exhibit the stage in which the embryo sac is ready for fertilization. C: mature seedling cell shows the polar nuclei and the remains of one of the synergites. D: shows Intersecting cells and Nousl in the Shalazi zone. Ap: Anti-pedal, cz: Shalazi zone, e: seelding cell, es: Embryonic bag, fu: Bond, ii: Internal shell, m: Microspor, mp: Microfiber, ob: Ovary, oi: Outer shell, Ov: ovule, pn: polar nuclei, sy: synergy).

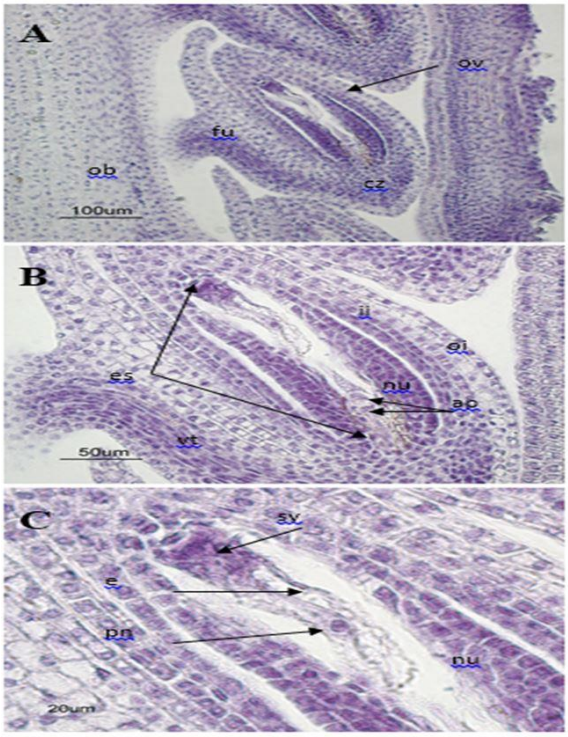

Figure 4: The longitudinal cut of the juicy flowers is colored with hematoxylene (A, B: shows the ovem and embryo sacs in the fertilization stage. C: it shows polar nuclei, the cells of the nucleus, and the remains of one of the synergites. Ap: Anti-pedal, cz: Shalazi zone, e: seelding cell, es: Embryonic bag, fu: Bond, ii: Internal shell, m: Microspor, mp: Microfiber, ob: Ovary, oi: Outer shell, Ov: ovule, pn: polar nuclei, sy: synergy, vt: Vascular tissue).

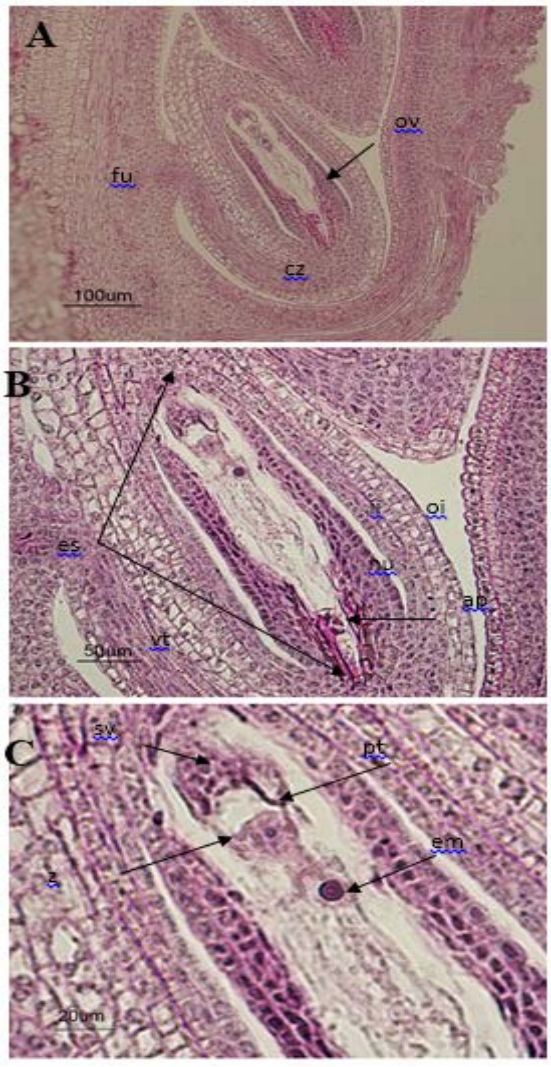

Figure 5: The longitudinal cut of the juicy flowers is colored with hematoxylene-PAS. (A, B: shows the ovem and embryo sacs in the fertilization stage. C: it shows the egg cell, the endosperm mother cell and the remains of one of the synergites. cz: Shalazi zone, em: endosperm mother, es: embryonic sac, fu: strap, ii: internal shell, oi: outer shell, ov: egg, pt: pollen tube remnants, sy: synergy, vt: Vascular tissue, z: egg cell).

Based on the provided cutting of 4 mm long microscopes (Figure 6), divisions in the basal cell occur earlier than the apical cell. The basal cell is divided into horizontal walls and creates a string of cells that form the suspensor. As seen in Figures 3-9, the basal cell suspensor is strongly vascular and coarse. Suspensor has many important tasks, but the permanent part is not in the embryo's building and will soon be degenerated. Regarding Figure 6B, endosperm cells in the microfiber pole have a dense cytoplasm that decreases their cytoplasmic density toward Shalaz.

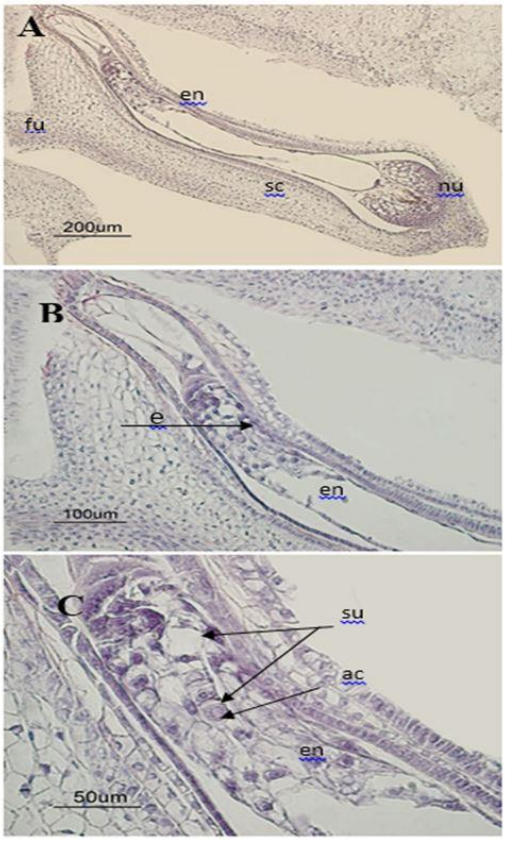

Figure 6: Longitudinal incision of 4 mm long Meriacraps colored with hematoxylene: (A, B: it shows the continuation of the formation of the wall around the endosperm free nuclei in the microfiber poles. C: it shows the divisions that are in the basal cell and the suspensory form. ac: apical Cell, e: Embryo, en: Endosperm, nu: Nousl, sc: Grain Shell, su: Suspensor).

Research results show that as a result of 3 sequential divisions in the egg cell and 8-cell pre-embryo is produced. The cell wall produced as a result of the third division is maintained as a specific area within the embryo at a later stage (Figure 7C). At this point, 8 cells are placed on the 2nd 4th floor. The cells of Shalazi pole are upper tier and the lower poles are lower tier. Botanists say that in start of the formation of cells in the upper and lower levels would be determined and from the very beginning of the Spherical embryo or the 8th cell embryo in the upper and lower floors would be specified. This stage is very important in the development of the embryo, because the subsequent division with tangential walling leads to the production of ancestral cells of the embryonic epidermal called protoderm. In this image, the suspensory cell is adjacent to the embryo is the pituitary cell. This cell plays a role in the formation of a cap and a quiet area in the plants.

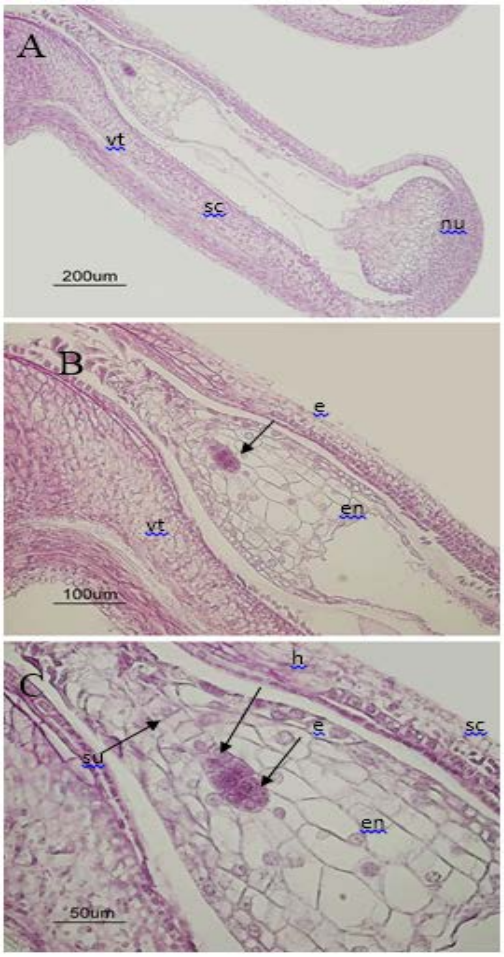

Figure 7: Longitudinal incision of 5 mm length Meriacarps colored with hematoxylin-PAS: (A, B, C: It shows the formation of 8-cell pre-embryo and the continuation of the development of endosperm around the embryo. In Figure 7A, there is an increase in the number of noucl cells in the Shalazi pole. e: embryo, en: endosperm, h: pituitary, nu: Noucl, sc: grain shell, su: suspensor, vt: vascular bundles).

Histologically, the root end of the meristem is located on the embryo's microfiber pole and the meristem of the stem ends is located on the opposite side of the root. End meristems of stems of dome-shaped, lacking in storage, have large-cellular cells. The meristem of the stem ends does not function in the Tribulus terrestris and leaves do not form a log. The meristem of the apical root that is located in the embryonic microfiber pole has been activated and several layers of the root cap cell have been obtained as a result of tangential divisions (Figure 8A-8D).

Histologically, hypocotyl and cotyledons, three groups of cells can be distinguished in them, which include:

- Protodermic cells that cover the surface of the embryo. These cells have a small size (Figure 8B).
- Parenchymal cells located below proto-dermal cells and are larger than protodermic cells. Below the abdominal protoderm, a layer of parenchymal cell differentiates as small and stretched cells under the protoderm and on the parenchymal cells of the cotyledon (Figure 8B).
- Prokambia cells are stretched and narrow cells. The prokambium cells in the longitudinal section are cylindrically, in the axial section of the hypocotyl and each of the cotyledons, which have been pulled up to the top of the cotyledon (Figure 8E). The splitting of these bunches occurs in the mid- section of the hypocotyl and the branching of the input to each clove is ramified and extended to all parts of the cotyledon.

The study of endosperm tissue in adult seeds shows that all endosperm cells have lost their contents at this stage, and only the thin walls of endospermic cells are observed which collapse in the course of cell growth. Endosperm is seen at this stage as lacy and very delicate structure, which is more developed and densely populated in the Shalazi pole and is stretched in the form of narrow strands on the sides of the cotyledons and the distance between them. These sections are visible after coloring with the PAS method to the purple. Nousl tissue in mature seeds is visible to small and dense cells in the lower and outer periphery and wrinkled cells in the upper part (Figures 8C and 8F).

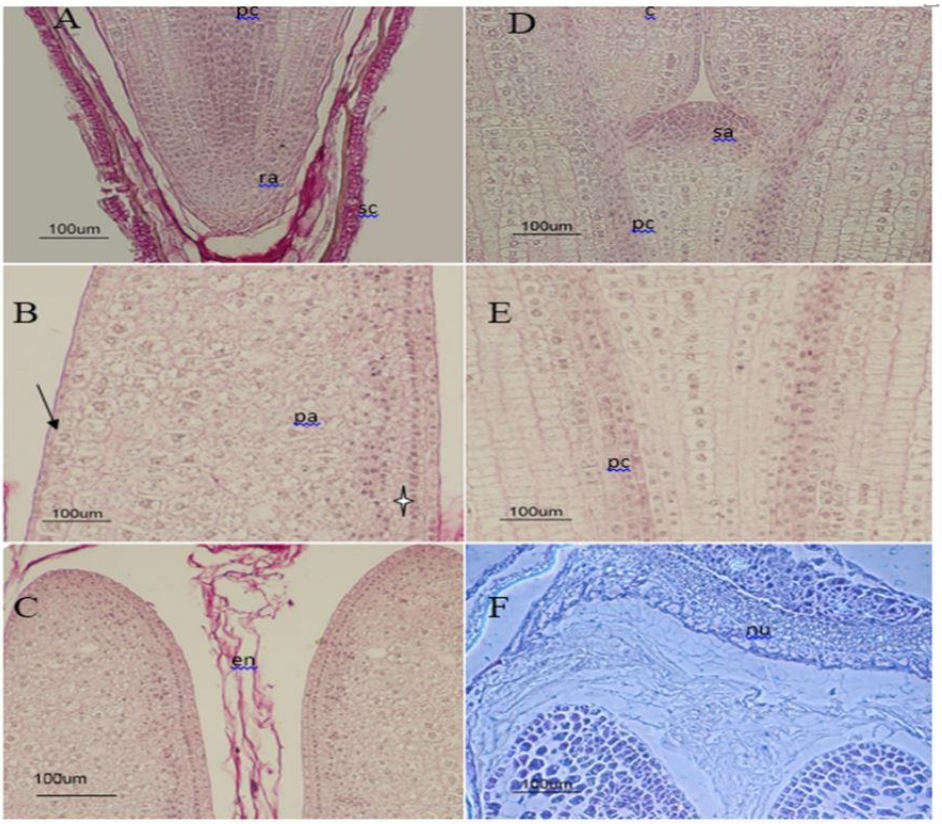

Figure 8: Longitudinal incision of adult embryo coloring with PAS (Figures 8A-8E) and Comasi Brilliant Blue (Figure 8C). (A, D: it shows apical Meristem of roots and stems of the adult embryo. B, E: It shows the longitudinal cut of the cotyledon, the hypocotyl and the protoderm arrows, and the parenchymal fence star. C, F: it shows Endosperm lacy structure and a very delicate and impure mature seed. c: cotyledon,: nu Noucl, en: endosperm, pa: parenchyma, pc: prokambium, ra: root meristem, sa: stem meristem.

Gradually, with the growth of cotyledons, the accumulation of starch-like storage materials begins. These materials are replaced by protein substances in the adult embryo, and the mature embryo contains very coarse protein substances with one or more proteins in the globin species (Figure 9).

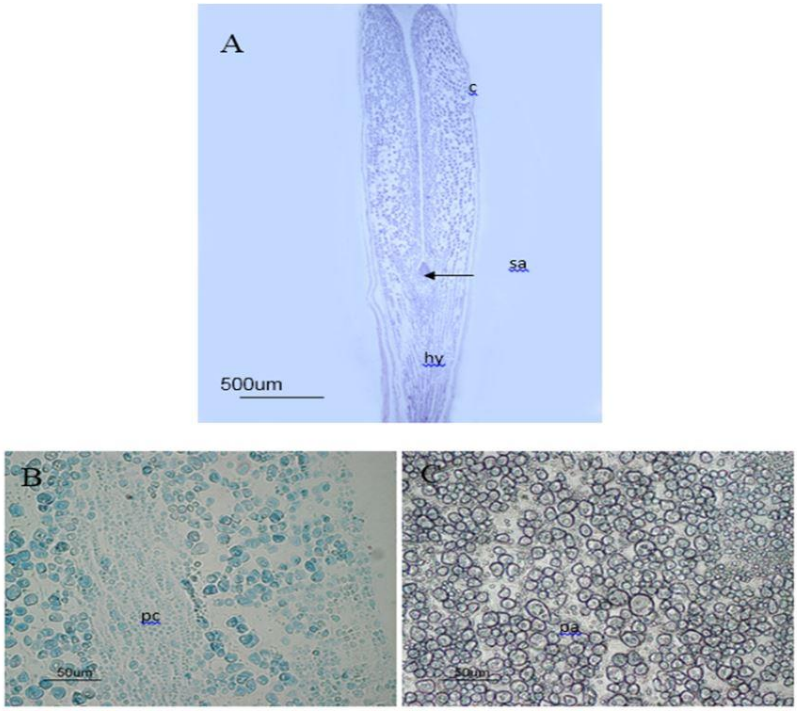

Figure 9: Longitudinal incision of adult embryo colored with Comasi Brilliant Blue (A, B) and black Sudan (C). (A, B: it shows the accumulation of protein substances in the adult embryo. C: it shows the range of protein substance after coloring with black Sudan. C: Cotyledon, hy: Hypocotyl, pa: Parenchyma, pc: Procumbium, sa: Meristem stem).

Protein substance are one of the most commonly used storage media for seeds, and many embryo and endosperm can be seen in vegetable spin. Structurally, protein bodies are composed of a protein matrix and another part called globoid or crystalloid and the matrix portion of which is a nitrogen-retaining source for the plant, and the globoid portion is the source of the element that acts as an insoluble salt of phytine within the protein matrix One or more numbers are visible. These objects are surrounded by a phospholipid membrane and will be enclosed by some lipids in some plants (Dourado et al., 2000). Due to the difference in the color intensity of different embryo organs in Figure 9A, cotyledons have the highest protein accumulation. Parenchymal cells have larger protein substance with more densities. While in proteodermic and Procumbium cells, protein bodies are seen with fewer and smaller sizes. The protein beads can be seen as blue in Figure 9B. The protein substance boundaries are more easily distinguishable after coloring with Black Sudan (Figure 9C).

Discussion and conclusion

The study of embryonic and endosperm development in various plant genes is very important in phylogenetic and systematic studies. In 1989, Tobe used 50 symbiotic attributes to examine systematic situations and evaluate evolutionary relationships in the Myrtle's order. He examined 48 traits for each species, including embryogenesis, type of embryo, susceptibility, presence or absence of impacted tissue in adult seeds, endosperm type, presence or absence of mature seeds, germinal development and etc. He concluded that systematic studies are possible by studying and the evidence of embryogenesis and other available evidence. Even when other evidence is not available or there is no consensus for solving a systematic problem, only embryonic traits can provide a solid and logical basis for solving such problems (Tobe, 1989). Significant morphological differences between the Harmal spin make it doubtful to put all thesematerials in one spin. In a study by Decraene (1996) on the genus Nitria and in 1996 on the genus Harmal and the differences between these materials were noted with other Harmal spin species. He used 42 morphological and anatomical traits and some embryonic features. The results of this study indicate that the type of embryogenesis is based on Johnson's classification of Anagrad type (according to the first division of the egg cell and the participation of the basal cell in the formation of the embryo). In this type of embryogenesis, the first division of the egg cell is horizontally and the prefect of the cell is T-shaped, which is derived from the derivatives of the apical cell. Suspensor and pituitary are also derived from basal cell derivatives. Histological studies have shown that cell divisions occur in the basal cell faster than the apical cells, and a 3-cell suspensor is produced, while apical cell is still not divided. The first division of the apicalcell occurs when the suspensor has 5 cells. In general, the study carried out in this study alone can't be used to determine the taxonomic status of Tribulus terrestris. Therefore, the combination of this research with more extensive investigations of morphological and anatomical traits and other embryological traits seems necessary to determine the taxonomic range and other phylogenetic studies of this genus in the Ziguiflace spin.

Research suggestion

Regarding to the results of this research, it is suggested to consider the following issues in future research:

- Ultra structural study of endosperm development in *Tribulus terrestris.*
- Ultra structural study of Noucl and Determining the exact role of it in plants.

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