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Long-term Effect Different Concentrations of Zn $(NO_3)_2$ on the Development of Male and Female Gametophytes of *Capsicum annuum* L. var California Wonder

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Abstract. Pepper is one of the most important crop plants. Recently, the global need for this plant has been widely increased due to its use in the food and pharmaceutical industry. we invested the effects of different concentrations of zinc on the development of male and female gametophytes of bell pepper (*Capsicum annuum* L. var California Wonder). The plants were cultivated with different concentrations of zinc nitrate (0 (control), 2.5, 5, 7.5, 10 and 15 mM) in a greenhouse under experimental conditions. Buds and Flowers are harvested at different stage of development (in 6 sizes) from May to Jun. They were fixed in FAA and maintained in 70% alcohol, then embedded in paraffin, sliced with a microtome and analyzed using a light microscope. Microscopic studies showed that developmental process of ovule, gynoecium and pollen grain in bell pepper plants was taking to ordinary process in dicotyledonous plants. According to the results, increased zinc concentration resulted in a disorder in the reproductive phase, which caused the treatment 1 to enter the reproductive phase with a 4-week delay. In addition, the other of the treatments did not enter the reproductive phase and were wilt during the growth period. The developmental stages of gynoecium and anther in treatment 1 were similar to the control. However, a number of abnormalities and irregularities wereo bserved including signs of nuclei disintegration, deformation of embryo sac, accumulation of dark materials and deformation of pollen grains.

Keywords. Bell pepper, male gametophyte, female gametophyte, zinc.

INTRODUCTION

Heavy metals refer to a group of elements with a density of more than 5 gr cm^{-3}. A few of them (Co, Fe, Mn, Mo, Ni, Zn and Cu) are essential micronutrients, which are necessary for normal growth, oxidation and reduction reactions, electron transferring, and other important metabolic processes in plants (Rai *et al*., 2004). Increase in zinc occurs mainly due to the environ-

mental pollution following industrial and agricultural activities such as smelter and incinerator emissions, spreading from mine wastes, excessive use of chemical fertilizers and zinc-containing insecticides, using sewage (waste water), sludge or other industrial and mineral fertilizers contaminated with zinc (Pedler *et al*., 2004; Giuffré *et al*., 2012). As it is rapidly absorbed by plants, it can be very toxic. Growth inhibition is a common phenomenon attributable to poisoning with zinc in plants. The more poisoning occurs, the less the product will become, and it eventually overcomes the growth and inhibits it (Broadley *et al*., 2007; Marschner, 2012), which is mainly because of the degradation of the photosynthesis activity. This affects photochemical reactions (Assche and Clijsters, 1986), carbonic anhydrase activity (Ló pez-Millán *et al*., 2005) biosynthesis of chlorophyll (Assche and Clijsters, 1986) and the integrity of the cell membrane (Wissemeier and Horst, 1987). If the concentration of zinc becomes higher than the critical level, it will lead to a decrease in growth and no flower production (Rout and Das, 2003). Pepper is one of the most important crop plants. The need for pepper cultivation has doubled over the past 20 years (FAO, 2017). Given the excessive increase in chemical fertilizers in agriculture, the increase in the amount of heavy metals in the environment, and the economic and nutritional importance of the pepper in recent decades in the world, the study aimed to examine the effect of zinc nitrate on anther and gynoecium development in this plant.

MATERIALS AND METHODS

Seeds from *Capsicum annuum* L. var California Wonder were provided from the plant gene bank of Seed and Plant Improvement Institute of Karaj, Iran. After sterilizing the seeds, they were cultured in a sterilized soil (obtained from Behkam Company). Six treatment groups with different concentrations of zinc (0 (control), 2.5, 5, 7.5, 10 and 15 mM) were selected and used for irrigation from the first irrigation until the end of the growth period. During the propagation stage, temperature was 25 ± 2 ° C, humidity 75-80%, and 16 h day light. Buds and flowers in all stages of development (in 6 sizes) were collected every week from May to Jun, 2017. The collected plant materials were fixed in a FAA (37% Formalin- Glacial Acetic Acid-70%Alcohol, 2:1:17 v/v) for 12 hours, then were stored in 70% alcohol and dehydration in an ethanol series and embedding in paraffin, specimens were sliced by Shandon AS325 rotary microtome. Staining of serial sections of 6-7 µm was carried out Hematoxylin- Eosine. Specimens were viewed with a OLYMPUS model BX43 light microscope connected to OLYMPUS digital camera. At least 15 flowers were observed for each developmental stage and the best samples were chosen for photographs.

RESULTS

Generative meristem and flowering

From the fifteenth to sixteenth weeks from the first day of cultivating, the generative meristems started their activity in the control plants and the buds emerged. In order to study the stages of flower development, the buds were considered in six stages: developmental stage 1: 1 to 2 mm diameter; developmental stage 2: 3 to 4 mm diameter; developmental stage 3: 3 to 5 mm diameter with the corolla hidden in calyx; developmental stage 4: 9 to 10 mm diameter corolla is in calyx; developmental stage 5: semi-open corolla with a 10-15 mm diameter; and development stage 6: approximately 20 mm in diameter the mature flower was considered with distinct corolla (Figure 1).

After the plant reaching the stage of flowering, the vegetative meristem is transformed into a generative meristem. Figure (2A-E) shows the developmental stages of generative meristem and the formation of different part of the flower. The generative meristem has a greater volume and densely stained compared to the vegetative meristem, which is the result of increased of mitosis activity in the apical meristem, tonica and corpus regions. Microscopic studies of generative meristem show that this meristem is enlarged and expanded and its stainability is almost homogeneous in different parts. Their cells are homogeneous, dense and more stainable compared to vegetative meristem. The terminal part of this meristem is sporogenous meristem (Sp.m) and its

Figure 1. Flower bud sizes: Stage 1: 1-2 mm diameter (St1). Stage 2: 3-4 mm diameter (St2). Stage 3: 3-5 mm diameter (St3). Stage 4: 9-10 mm diameter (St4). Stage 5: 10-15 mm diameter (St5). Stage 6: 20 mm diameter (St6).

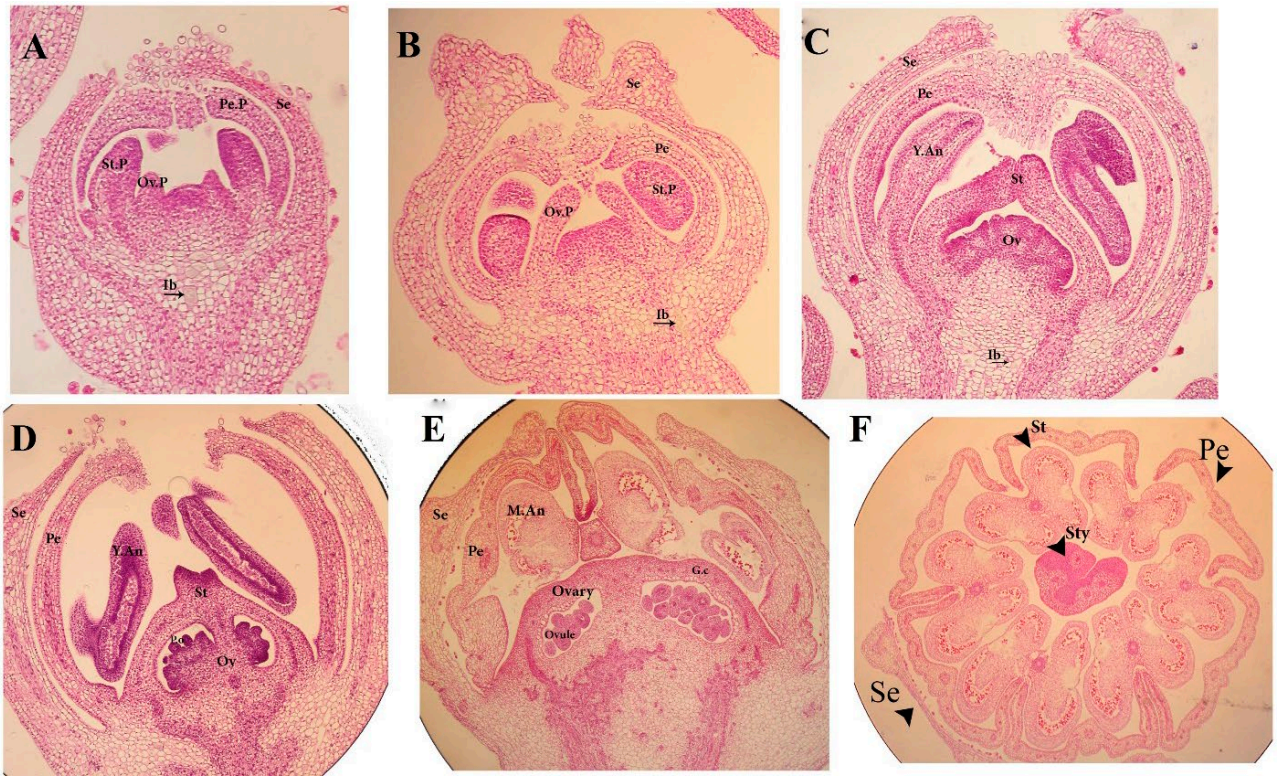

Figure 2. Longitudinal section of floral bud in different stages of flower development: (A): Stage 1. (B): Stage 2. (C): Stage 3. (D): Stage 4. (E): Stage 5; (F) Transverse section of floral bud (Ib – Idioblast; M.An – mature anther; Ov.p – ovary primordium; P – Petal; Pe.P – Petal Primordium; P.o – ovule primordium; Se – Sepal; St.p – stamen Primordium; St - stigma; St: stamen; sty – style; Y.An – young anther).

lower part, which is less stainable, is called receptacle meristem (Re.m). With the formation of generative meristem, the structural components of the flower such as Sepals (Se), stamen primordia (St.P), and petal primordia (Pe.P) gradually become distinct (Figure 2A). Simultaneous to the formation of stamen, petals (Pe) are formed, as a result of the activity of the peripheral part of sporogenous meristem against the stamen, and in the final stage, the ovary primordium (Ov.p) is formed (Figure 2B). Sepals (Se) appear with the activity and the remaining divisions of the initial ring (Figures 2A-B).

In the next steps, from the outside to inside, Sepals (Se), petals (Pe), stamen primordia (St.P) and ovary primordium (Ov.P), can be distinguished, respectively. The proliferated and deformed mass of the ovary primordium indicate the beginning of the ovary (Ov) and style (St) organization (Figure 2 B-C). Style, ovary, and ovules primordia were formed in the gynoecium. Following the mentioned changes and with gradual development of flower, sepals, petals, stamens, gynaecium, and ovules primordia can be detected (Figure 2D), and the full flower structure with mature stamen and carpel is shown in Figure 2E.

Microsporogenesis and pollen grains development

Bell pepper (*Capsicum annuum* L.) flowers are bisexual (Figure 2C-E). The flower is hexamerous (Figure 2F). All the stamens are equal in size, and they surround the style and the stigma. In addition, the anthers are tetrasporagiate and contain four pollen sacs (Figure 3 A-D). First, the stamens primordia are formed, then in the middle part, the pistil primordium is formed. As seen in Figure 2B, the development of the stamens is faster than the gynoecium and ovule. When the pistil primordium is still seen in form of cell masses without any differentiation, the stamens (filament, anther, and various layers of the anther wall and the cells of the sporogenous tissue) (Figure 2A-B) are observed and recognized clearly.

The young anther cell wall contains an epidermis, endothecium, two middle layers and tapetum from the outside to the inside (Figure 4A). Young anther layers wall cells have the same size and their large nuclei occupy most of the cell volume (Figure 4A). A series of rectangular cells form a row of single nucleus epidermis (Figure 4A). Before maturation, endothecium forms a row of single-nucleus rectangular cells (Figure 4B).

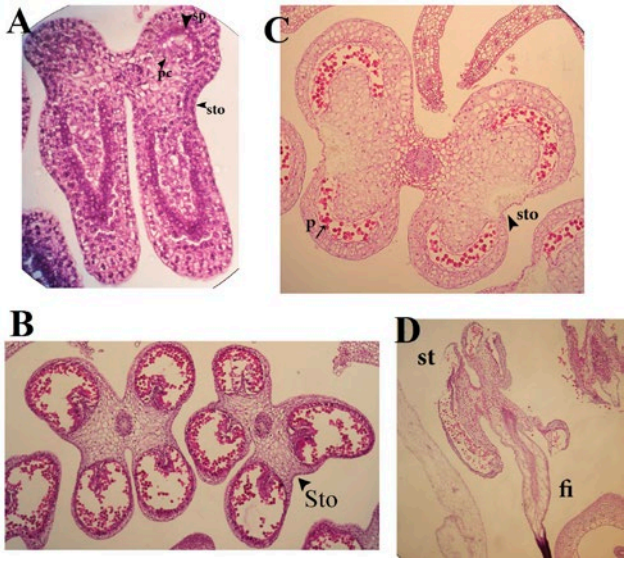

Figure 3. Transverse section of anther in different stages of development. (A) Young anther; (B) Second meiotic division in anther; (C) Anther at the binucleate pollen grain stage; (D) Longitudinally dehiscent anther in cross section (fi – filament; p – pollen; pc – placentoids; sp – primary sporogenous layer; st – stamen; sto – stomium).

During maturity, these cells are radially divided, and the single- double outer endostium form up to 3 layers of cells towards the connective tissue (Figure 4C-D). At this time, these cells form thick fibrous and the middle layers are decomposed (Figure 4C-D). First, the tapetal cells are secretive single-cellular (Figure 4A). The nuclear division in these cells usually occurs before the onset of meiosis in the pollen mother cells (PMCs) and they become bi-nuclei (Figure 4C-E). During development of the anther, these cells develop radially and they are filled with dense materials (Figure 4C). Tapetal cells differ in the morphology characteristics: on the outer surface of anther wall, they are stretched, rectangular and relatively uniform, whereas they become longer radially towards the connective tissue and are hypertrophied (Figure 4F). Sporogenous cells of anther have a dense stainable cytoplasm their large nuclei occupy most of the cell volume (Figure 5A). First, they are placed next to each other, then separated from each other and differentiated into PMCs (Figure 4A-B). First, to create pollen grains, these cells have to undergo a meiosis, but before initiation of the division process of PMCs, secrete a spe-

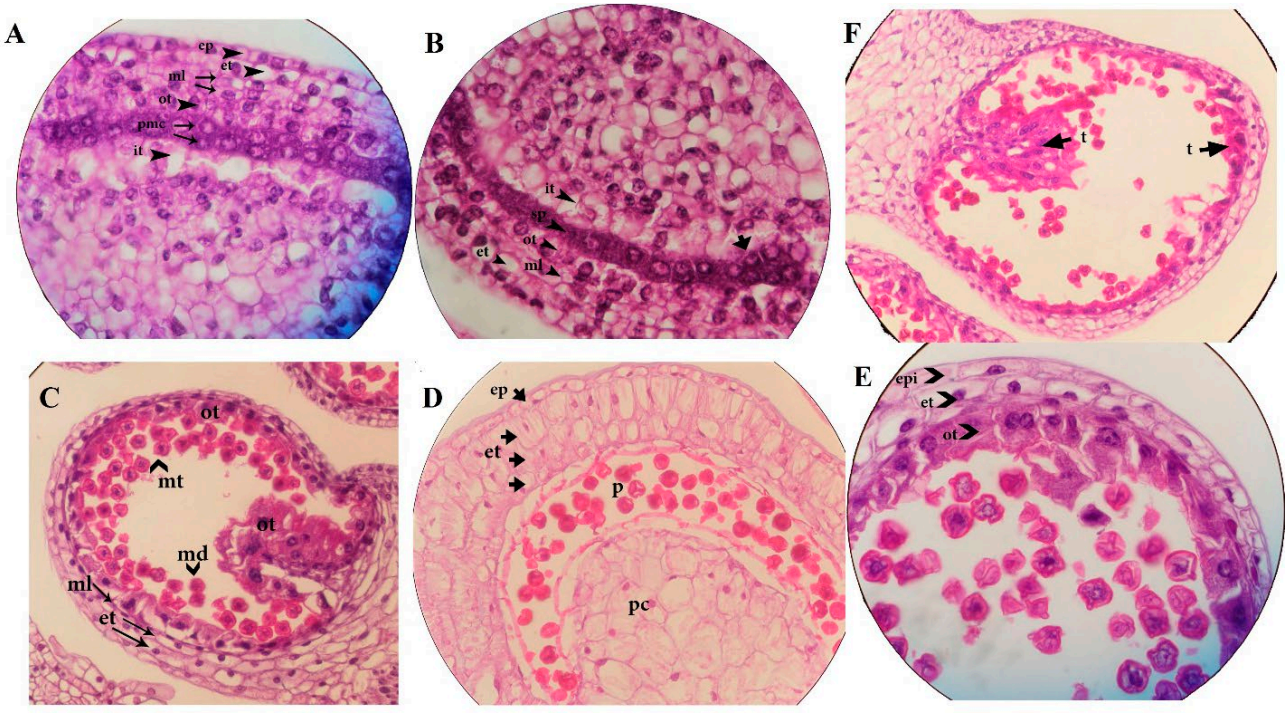

Figure 4. (A) The young anther wall layers and bilayer sporogenous tissue; (B) Anther sporogenous tissue is forming; (C) Degenerating middle layers and dividing endothecium cells at a single locule of anther. (D) Transverse section of a single locule of mature anther before dehiscence showing degenerating intersporangial septum and anther wall with only epidermis and endothecium; (E) Two-nucleated glandular tapetal cells on the outer side of anther wall; (F) Transverse section of microsporangium showing outer and inner binucleate tapetal cells, disintegrating callose in microspores tetrads and anther wall layers (et – endothecium; ep – epidermis; it – inner tapetum; ml – middle layer; mt – microspore tetrads; md – microspore diad; ot – outer tapetum; pmc – pollen mother cell; p – pollen grain; pc – placentoids; t – tapetum).

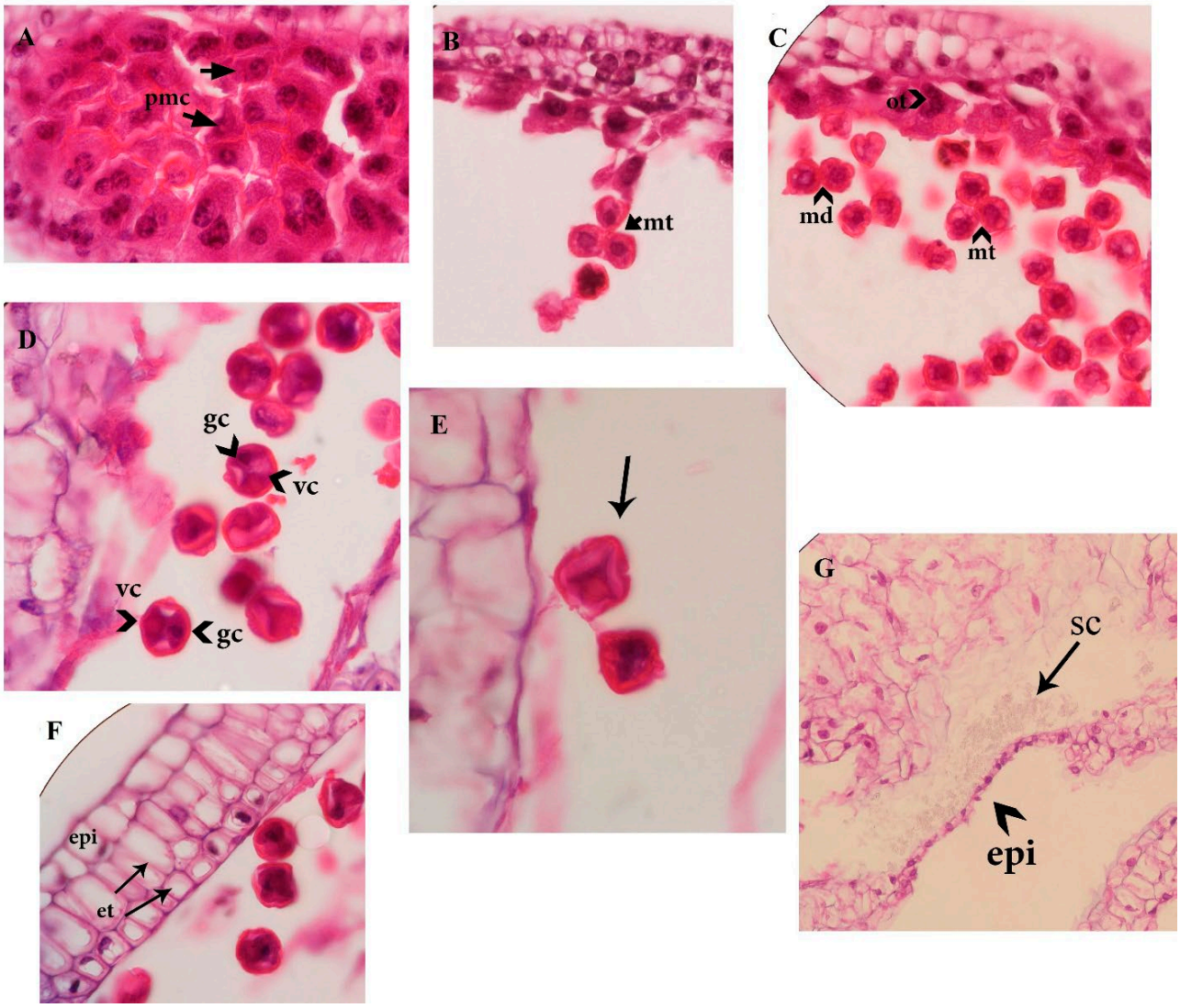

Figure 5. (A) pollen mother cell; (B) Transverse section of anther with microspore tetrahedral tetrads; (C) Microspore tetrads and diads stages. (D) Binucleate pollen grains. (E) Tricolporate pollen grains. (F) mature anther before dehiscence showing anther wall with only epidermis and endothecium; (G) Stomium degenerating epidermis cells and anther dehiscence (ep – epidermis; et – endothecium; gc – generative cell; md – microspore diad; mt – microspore tetrads; ot – outer tapetum; pmc - pollen mother cell; sc – sand crystal; vc – vegetative cell).

cial callose wall around them, surrounding the whole cell (Figure 5A). After the meiosis and the formation of the dyad (Figure 4C) and then the tetrad (Figure 4C, 5B), when the callose is decomposed, all microspores are released from tetrad into the lucule (Figure 5C). They have dense cytoplasm and thin cell walls (Figure 5C-E). At the end of the single nucleus stage, the microspore nucleus is pushed to one side. Following the expansion of microspore, the nucleus is divided and two dissimilar nuclei (generative and vegetative) are produced (Figure 5D). Mature pollen grains normally have the large vegetative cell and a small generative cell (Figure 5D). At this stage, only the epidermis and endostium exist in the anther wall (Figure 5F). Anthers dehiscence occurs through a longitudinal gap in the stomium (Figure 5G). It is formed by a layer of at most 20 cells under the epidermis, which is highly stainable and contains calcium crystals (shown with an arrow on Figure 5G).

Gynoecium, ovule and embryo development

In bell pepper (*Capsicum annuum* L.) the gynoecium is syncarpous, and the ovary is superior and

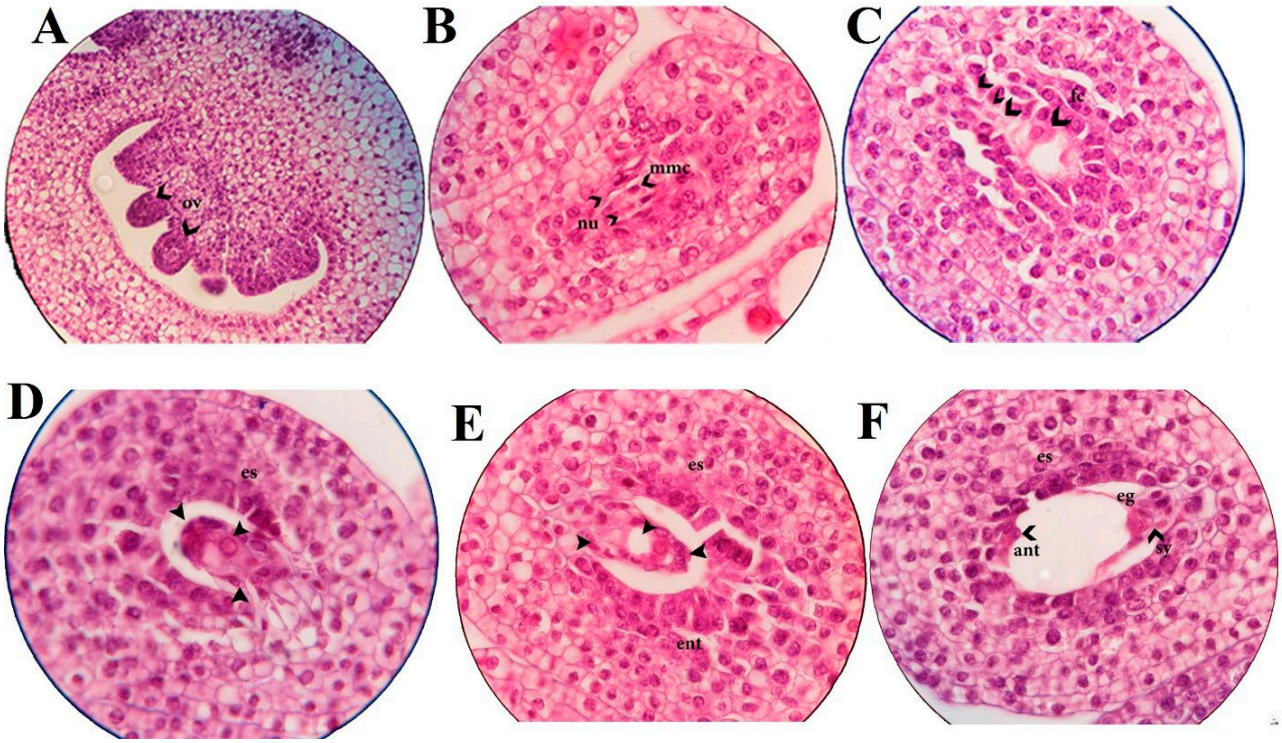

Figure 6. (A)Section of a single locule of ovary; (B) Megaspore mother cell (mmc) beneath the apical epidermis of the nucellus; (C) End of the mmc second meiosis and linear arrangement of the megaspores; (D) Eight-nucleate immature embryo sac; (E) Eight-nucleate immature embryo sac; (F) Eight-nucleate and seven-celled mature embryo sac (ant – antipodal; eg – egg cell; ent – endothelium; es – embryo sac; fc – functional megaspore; mmc – megaspore mother cell; nu – nucellus; ov – ovule; sy – synergid).

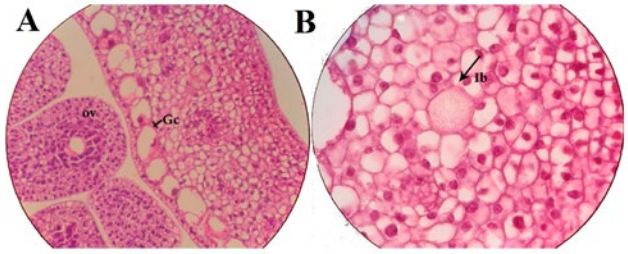

Figure 7. (A) Transverse sections of ovary wall with giant cell; (B) Transverse sections of ovary tissue with idioblast (Gc – giant cell; Ib – idioblast; ov- ovule).

ring shaped. The ovary is trilocular and consists of two carpels. Each carpel has a large number of ovules on the placenta axis (Figure 6A). The ovule is unitegmic, hemianatropous and tenuinucellate (Figure 6B). The embryo sac in the ovule is of polygonum type. The uninucleate archeospore is developed under the apical epidermis of the ovules and acts directly as a primary sporogenous cell. Thus, the meiosis in the megasporcyte initially forms as dyad and eventually as a linear tetrad (Figure 6B). In the tetrads, the sister megaspores are gradually decomposed, and the development of the embryo sac begins with the chalazal megaspore (Figure 6B). Three mitosis divisions continuously produce functional megaspore in the embryonic sac of two, four, and eight nuclei (Figure 6C). Thus, the development of the embryonic sac confirms the polygonum type (Figure 6E). For the formation of an embryo sac, the megaspore volume increases and its nucleus is divided into two nuclei, migrating to each pole of the cell (chalaza, micropyle) (Figure 6D). Then large vacuole is created between the nuclei. The nuclei are divided two times, and four nuclei of haploid are formed on each pole of the embryo sac (Figure C-D). A nucleus migrates to the middle part of the embryo sac from each pole, polar nuclei are formed, and the remaining three nuclei evolve into three cells at each pole (chalaza, micropyle). There are three adjacent cells to the micropyle which are formed, the middle is called egg cell (oosphere), and the two symmetric lateral cells which are called synergid opposite to the chalaza, as well as three cells opposite to the micropyle which are called antipodal (Figure 6F). These cells have a short life and die before fertilization in the embryo sac. The giant cells underneath the inner epi-

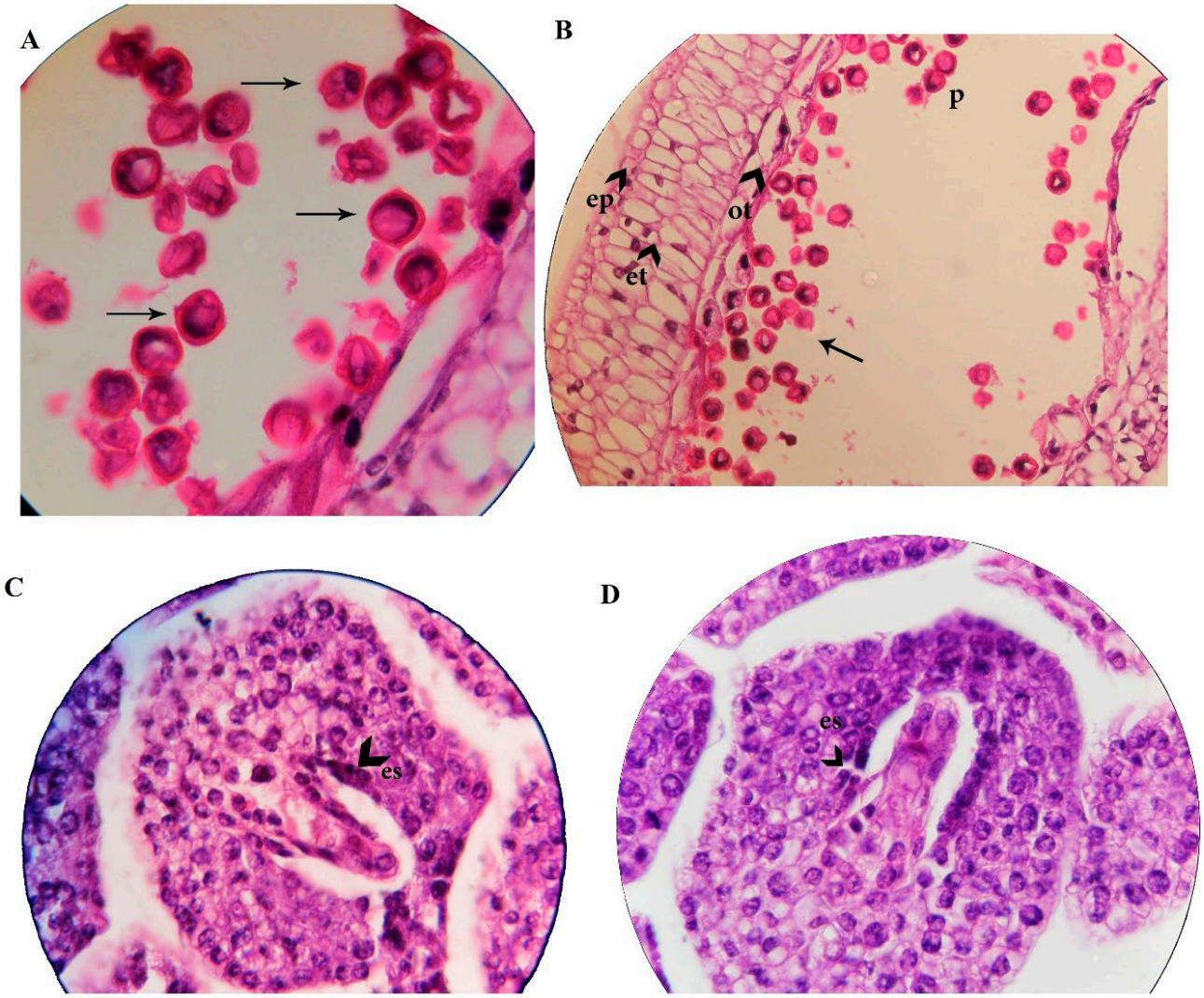

Figure 8. (A-B) Transverse sections of anther from treatment 1. abnormalities in pollen grains and collected black materials in pollen grain; (C-D) Transverse sections of ovule from treatment 1**.** Change of the shape of embryo sac and also degradation of egg apparatus in plants (et – endothecium; ep – epidermis; es – embryo sac; ot – outer tapetum; p – pollen grain).

dermis of the ovary wall are observed in developmental stages. During the developmental stages of the ovary, they enlarge (Figure 7A). Idioblasts containing calcium crystals are found in the ovule and ovary parenchyma cells (Figure 7B).

Changes pollen grain and embryo sac in treatments

In treatment 1 (2.5 mM), the plants entered the reproductive phase in the 19th to the 20th week with a 4-week delay. The plants in other treatment groups did not enter the generative phase (the number of culture periods was repeat three times, and no generative phase was observed). The process of male gametophyte development in this treatment was similar to the control. Pollen grains from zinc-treated plants showed that the shape of pollen grains was changed from the normal spherical state to a smaller shape, and a large number of pollen grains had irregular shapes. A large number of pollen grains showed the accumulation of dark materials (Figure 8A-B).

In the development of female gametophyte, the examination of microscopic slides in the plants under treatment showed signs of nuclear degradation and embryo sac changes (Figure 8C-D).

DISCUSSION

The studies on the structural characteristics of male and female gametophytes and the details of microsporogenesis and macrosporogenesis in various genera of solanaceace showed that bisexual are common in this family and dioecy are relatively rare (Davis, 1966), Hunzikera, 2001, Talebi *et al*., 2016, Ramezani *et al*., 2018). Microscopic studies showed that developmental process of ovule, gynoecium and pollen grain in bell pepper plants was taking to ordinary process in dicotyledonous plants. The differentiation of the various parts of the floral from the vegetative meristem was consistent with the results of Munting (1974) on *C.annuum*. The stamens in *C.annuum* are composed of relatively large anthers equal throughout the filaments. These stamens are tetrasporangiate and contain four pollen sacs (Dharmadhaj and Prakash, 1978), a distinct state in potato family (Endress, 1996). The taptum is glandular and its cells are binuclear. Secretive tapetum is observed in some Solanacease, such as *A. belladonna* (Yurukova-Grancharova *et al*., 2011), *W. somnifera* (Ghimire and Heo, 2012), *P. hybrida* (Chehregani and Ramezani, 2016), *S.tuberosum* (Talebi *et al*., 2016). In our observations, the structural characteristics of the tapetum cell layer were different from that of the outer surface of the anther wall and to the connective tissue in the bell pepper (*C.annuum*), that was consistent with the results of Yurukova-Grancharova *et al*. (2011) on *A. Belladonna*, Chehregani and Ramezani (2016)) on *P. hybrid* and Ramezani *et al*. (2018) on *C.annuum*. Although in these plants, the tapetal cells are different not only in structural but also in the number of nuclei, the number of nuclei in the tapetal cells is inconsistent in all families of Solanaceace (Tobe, 1989). As stated, for *A.belladonna* (Yurukova-Grancharova *et al*., 2011) it has four nuclei, for *W. somnifera* (Ghimire and Heo, 2012) two nuclei, for *P. hybrid* (Chehregani and Ramezani, 2016) four nuclei, and for *S.tuberosum* (Talebi *et al*., 2016) it has several nuclei.

Our results showed that tapetal cells of *C.annuum* had two nuclei, similar to *W. somnifera* (Ghimire and Heo, 2012) and *C.annuum* (Ramezani *et al*., 2018), whereas Dharmadhaj and Prakash (1978) reported three nuclei for *C.annuum*. At the time of maturation, each pollen grain of pepper has two nuclei; the presence of two-nucleus pollen grains is commonly found in Solanaceace (Davis, 1966, Dharamadhaj and Prakash, 1978, Poddubnaya-Arnoldi, 1976, Talebi *et al*., 2016, Ramezani *et al*. 2018). The sandy crystals accumulate in the anther cells of *C.annuum*. Crystals appear in the early stages of flower development in most solanaceace anthers (D'Arcy and Averett, 1996). When the *C.annuum* pollen grains are matured, the anther wall breaks down, and these calcium oxalate crystals are released (Ramezani *et al*., 2018). Our results confirmed the previous studies by the authors for different speices of Solanaceace (D'Arcy and Averett, 1996; Rezanejad, 2006; Chehregani and Ramezani, 2016, Ramezani *et al*., 2018). The released calcium crystals stick to the pollen and dissolve in the style mucous, and calcium ions can generate pollen for germination and pollen tube growth (Iwano *et al*., 2004).

The differentiation of male gametophyte in bell pepper (*C.annuum*) happens early from the female gametophytes, which is consistent with the results of Munting (1974) and Ramezani *et al*. (2018). The development of the embryo sac is done as a common pattern in all Solanaceace (Davis, 1966). In some *C.annuum* samples, we observed bilocular or trilocular ovary. Indeed, the bilocular ovary is a characteristic feature of Solanaceace (Ghimire and Heo, 2012, Ramezani *et al*., 2018). *C. annuum* ovule is hemiantropous that is consistent with the results of some varieties of *C. annuum* (Munting, 1974) and Ramezani *et al*. 2018 on the same variety. The present study showed that the development of the female gametophyte *C. annuum* L. var. California Wonder is of polygonum type. Polygonum type is typically known as Solanaceace (Mohan and Kamini, 1964, Mohan, 1966, Munting, 1974, Dharmadhaj and Prakash, 1978, Yurukova-Grancharova *et al*., 2011, Ghimire and Heo, 2012, Brito *et al*. 2015, Ramezani *et al*., 2018).

According to Van Assche (1986), high doses of zinc inhibit many metabolic activities in the plant by damaging the mitochondrial structure in cells (Rout and Das, 2003). The high accumulation of zinc in the cytosol of the plant cell also results in impaired cellular function and inhibition of respiration and energy responses associated with cell growth that might reduce the growth and development of the whole plant (Bonnet *et al*., 2000). This element affects the process of cell division by interrupting the interphase, prolonging the stage of prophase, G2, and stopping the synthesis of the synthesized proteins required by the cell cycle and nucleic acid synthesis (El-Ghamery *et al*., 2003). The continuity, integrity and permeability of the membrane are impaired due to toxicity of zinc. At the molecular level, it also affects the expression of many genes (Wang *et al*., 2009). Nutritional conditions play an important role in flower formation (Taiz & Zeiger, 2010). The zinc stress and increase in its concentration disrupt the plant's balance nutrition. On the other hand, the concentration of zinc affects hormonal balance and causes a disruption in hormonal balance and because hormones act as genetic regulators in inducing expression of the involved genes (Rout and Das, 2003). According to Aloni reports in 2006, delayed

flowering is due to a hormonal disorder such as auxin, which is due to gene disruption.

In treatment 1, the flowering began with delay and the rest of the treatments did not enter the reproductive phase, although stresses such as heavy metals typically cause premature aging of plants, and from the developmental perspective one of the symptoms of aging is flowering. However, in the interpretation of latency and lack of flowering in *C.annuum* seen under the influence of zinc stress in our study, one can state that the nutritional conditions of the plant plays an important role in the formation of the flower. Studying the C/N ratio in different plants has shown that each time a plant prepares for flower formation, this ratio goes higher (Taiz & Zaiger, 2010). Increase in the concentration of zinc causes disruption of absorption in other elements, including iron (Zeng *et al*., 2011), which causes a delay or lack of flowering.

Studies on microscopic sections of control and treatment samples showed that the general trend of ovule and pollen formation in plant treatment 1 and control was the same. However, for the plants in treatment 1, a large number of pollen grains smaller than the normal size were found. The results were consistent with Mohsenzadeh (2011) on *Reseda lutea* L., Yousefi (2011) about the effect of heavy metals pollution on *Chenopodium botrys* L., Albooghobaish *et al*. (2011) about the effect of lead toxicity on *Matricaria Chamomilla*, Rezanejad *et al*. (2003, 2007, 2008, and 2009) on the effects of air pollution on various plants, and Wolters and Martens (1987) on the effect of air pollution on pollen grains. Moreover, it was seen that in pollen grains that accumulated dark materials, which was also consistent with Chehregani *et al*. (2006) on the effect of acid rain on *Phaseolus vulgaris*. In treatment 1, a large number of ovules have deformed embryo sacs, so that compared to the control group, where embryo-sac has a spindle and stretched form, they have changed in their shape, which was consistent with the results of Hosseini (2006) on *Phaseolus vulgaris*.

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