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The Effect of NaCl on Anther Development in *Pistacia vera* L.

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Abstract

Plant growth and development are adversely affected by salinity. In order to study the effects of salinity on male gametophyte development steps in pistachio plant, an experiment was conducted in two areas in Golshan Anar with equal conditions: a control area (A) that was irrigated with fresh water well, and the other area (B) with salty water and EC values 14 dS.m-1 NaCl solution. A sampling of flowers was performed in two areas of Golshan Anar in the spring based on a completely randomized design with three replications. Male gametophyte development steps in pistachio plant were examined using conventional cell histology techniques and light microscopy observations and were then compared with samples subjected to no salinity stress. The results showed that some stages of male gametophyte development: (1) the anther undergoing normal development which is tetrasporangiate, (2) cytokinesis occurring simultaneously with meiosis in the microspore mother cell, the tetrahedral tetrads, (3) microspores being generated after meiosis by microsporogenesis were more or less irregular in shape during the contraction period. Finally, the abnormal shape and structure of the number of cases reviewed in three replicates of pollens studied can be one of the important factors affecting the decrease in the product.

Kayword: salinity, pistacia, anther

1. Introduction

Plant growth and development are adversely affected by salinity which is a major environmental stress that limits agricultural production (Hargurdeep, 1997). Reproductive development from meiosis in the spore mother cells to fertilization and early seed establishment is extremely sensitive to various stresses, such as drought, heat, cold, flooding, and nutrient deficiencies (Salter & Goode, 1967; O'Toole & Moya, 1981; Saini & Aspinall, 1981; Westgate & Boyer 1986 Satake & Yoshida 1978; Saini & Aspinall 1982a; Schoper et al., 1987; Morrison, 1993; Hayase et al., 1969; Brooking, 1976; Lardon & Triboi-Blondel, 1994, Matsushima, 1962; Reddy & Mittra, 1985, Zavadskaya & Skazkin 1960; Graham, 1975; Campbell & Leyshon, 1980; Sharma et al., 1987; Azouaou & Souvré, 1993). These stresses cause various structural and functional abnormalities in reproductive organs, leading to failure of fertilization or premature abortion of seed or fruit. Thus, the damage to productivity from stress at this stage is particularly severe for crops in which the economic yield is the product of sexual reproduction, as increase in water deficit probably ranks as the most important environmental factor limiting global crop productivity (Fischer & Turner, 1978; Boyer, 1982). Among these stresses, salinity is an old problem since the past centuries. About 20% of land under cultivation and almost 50% of irrigated lands in the world are affected by ion concentration-(Zhu Jk, 2002). High salinity has two damaging effects on plants. The first is resulted by the water shortage because of increasing the concentration of soil solution and the other is due to toxic ions which prevent enzymatic activity in key processes (Zhang & Blumwald, 2001). Plants employ several mechanisms to weaken the effects of these environmental stresses. One of these mechanisms is the defective formation of eggs or pollen that causes the transfer of the food sources from the reproductive organs into metabolic reactions and causes resistance to stress. Salinity stress can also stimulate or predate aging of the reproductive organs. Asch and Wopereis (2001) showed that salinity decreases the rice yields by 45% as a result of the sterility of clusters and the reduction of seed weight in formed seeds. In cotton, salinity is a major cause of defective grain and reduction of product and its quality (Davidonis, Johnson, and Landivar, 2000). The studies of Namuco (1986) and Westgate (1986) showed that microspores during plant growth are very sensitive to salinity. Also, according to Sun et al. (2004), in

Arabidopsis plants grown under salinity stress conditions, microsporocytes were not converted to the mature pollens grains rather, these cells became vacuole and mainly became old during two days. In addition, fallen anthers were full of the remaining pollen grains. Interestingly, mature pollen grains were not affected by prolonged periods of salinity stress, which proves the effect of salinity on the pollen grains depending on the growth developmental stages of anthers. Male reproductive development in plants is highly sensitive to water deficit during meiosis in the microspore mother cells (Hargurdeep, 1997).

Pistachio (Pistachio vera) belongs to the family Anacardiaceae and order Sapindales (AlSaghir, 2010). Based on morphological characteristics, pistachio (P. vera) is known as the oldest species of this genus (Baninasab and Mobli, 2008). P. vera is a dioecious woody plant with imparipinnate leaves that fall in autumn (AlSaghir, 2010). The male and female inflorescences have 450-500 and 150-250 flowers, respectively. The height of this tree may reach up 7-10 meters and it has a very long lifespan (Asaja, 2006). When the female flower opens, stigma surface is receptive to pollen, and fruit and seeds are produced usually after successful pollination. Female inflorescence bud located on a one-year woody branch begins to swell on late March and during the first two weeks of April; 100 to 300 flowers are pollinated within each inflorescence (Polito & Kallsen, 2005).

Morphological and developmental studies on female flowers and embryogenesis in the genus Pistacia L (AlSaghir, 2010; Bachelier & Endress, 2007; Grundwag, 1976) and the species P.vera (Lin, Crane, & Polito, 1984; Martinez-Palle & Herrero, 1998; Shuraki & Sedgley, 1996; Shuraki & Sedgley, 1997) have been conducted by different researchers. For example, Endress and Bachelier (2007) investigated genus Pistacia L. and reported that female flowers have 5-8 calyx-like excrescences; the female part contains a large spherical ovary with a short style and trifurcation of stigma (each branch has two lobes). Grundwag (1976) reported that this genus has one ovule per ovary, downward-directed ovule, monolayer, and a large amount of nucleus. The embryo sac in the genus (Grundwag, 1976) and species (Lin, Crane, & Polito 1984) has Polygonum-type. Nevertheless, there are few studies on male flowers (Al-Saghir, 2010; Asaja, 2006; Azouaou and Souvré, 1993; Bachelier and Endress, 2007; Shiyan, Jain, and Mijiti, 2001; 25- Xu-Xin et al., 2011; Zeng-Fang et al., 2010). Most of these studies have been conducted on morphological structure, and less attention has been paid to flower development in pistachio. Different researchers conducted morphological studies on pistachio pollen and reported that its morphology is diversified among different varieties (Afshari et al., 2008; Davarynejad et al., 1996; Xu-Xin et al., 2011; Zeng-Fang et al., 2010). Pistachio seeds have a special economic importance in the family Anacardiaceae, genus Pistacia L. Based on Molecular Data Bank of Iranian Pistachio reported in 2008, pistachio is the first non-oil exported product in Iran (IPMD). Several methods and studies have been conducted to develop the knowledge about this valuable species as well as to distinguish differences among different varieties. However, the morphological and developmental assessments of flowers, particularly male flowers, are very limited. The key characteristics of reproductive organs and studying the developmental stages of gametophytes have a great importance in botanical sciences since they are appropriate tools for the identification and classification of plants. the present inquiry set to study anatomical characteristics of male flower and developmental stages of anthers in pistachios and the effect of salinity on these stages in natural condition without entrance of any natural and experimental factors. Biological stress of pistacia was studied by Seydi et al. (2015), Parsa and Karimian (1975), Ranjbar et al. (2002), Chelli-Chaabouni et al. (2010), and Bastam et al. (2013).

2. Materials and Methods

2.1 Plant Material

This comparative study was conducted on two areas with equal conditions including a control garden(A) that was Irrigated with fresh water well and the other garden(B) with salty water well with EC values (electric conductivity) 14 dS.m-1 NaCL solution. A sampling of flowers was performed in two gardens of Golshan Anar area in the spring based on a completely randomized design with three replications. Golshan is a village located around Anar in the suburb of Rafsanjan city, Kerman Province. The study area is 14 km southeast of Anar, with a longitude of 55º21′, the latitude of 30º48′, and the average height of 1408 m with a desert and hot-dry climate. To determine the soil texture, soil samples were first collected from the surface up to a depth of 120 cm and the percentage of each of the three sand, silt, and clay particles were analyzed in soil samples in the soil laboratory of Rafsanjan's Pistachio Research Center. Then, using the soil textural triangle, the percentage of sand was determined in table 1. (Mohammadi, 2006).

Table 1. Soil particles percentage and type of soil texture

Soil particles				Area of Pistachio
Type of soil texture	Sand (%)	Silt (%)	Clay (%)	
Sandy loam (light)	54.6	25.6	19.8	A
Sandy loam (light)	62.6	17.6	19.8	B

2.2 Anatomical Studies

To study the developmental stages of anthers, sampling from male inflorescence buds was done until the opening of anthers and pollination. Morphology of inflorescence and male flowers was investigated using a dissecting microscope. For the anatomical and developmental study, the samples were fixed via FAA solution (90% ethanol 70 + 5% acetic acid+ 5% formaldehyde) for 24-72 hours(Johansen, 1940), and then placed under running water for 24 hours. The samples were dehydrated in a series of increasing ethanol concentrations and then fixed in alcohol 70%. For paraffin embedding, the samples first were hydrated using ethanol 70, and then the ethanol in tissues was gradually replaced with toluene (paraffin solvent) by soaking the samples in solutions with increasing toluene level for 20 minutes. Next, molding was performed on samples placed into molten paraffin for at least 7 hours. Sectioning was conducted using rotational microtome. The slides were deparaffinized by toluene and. were Stainined with Hematoxylin- Eosin according to the protocol suggested by Meyer (Yuang, 1984). Sectioning with a microtome.The thickness was 0.8 micrometers To adhere the samples, the sections were first dehydrated in distilled water and increasing ethanol series and until they became transparent in toluene. Finally, the permanent slides were obtained using Entalen glue and coverslip. Microscopic examination of samples was conducted under a light microscope (Olympus BH2 Japan) and the appropriate samples were photographed using a digital camera (Canon IXUS 120 IS USA).

3. Results

Morphological and anatomical study of male flowers showed that male inflorescences are formed in the form of complex and lateral panicle on the branches before the appearance of the leaves (Figure A1). At the flowering period, the color of anthers is red that changes during developmental stages. At the pollination time, they are completely yellow or their pedicel is yellow, but the tip remains red. Anthers color change begins from the ventral surface of inflorescence and reaches to the dorsal surface (Figure A 2, 3). Male flowers have four to six stamens with large and bulky anthers, two or three sepals and 0-1 bract; the sepals and the bracts are distinguishable based on size. Filaments are short and bulky and dehiscence of anthers occurs in length and depth close to the central vascular bundle of anthers (Figure A 4-6). Cross sections of male inflorescence confirmed the number of anthers per flower that the four-anther was dominant (Figure A 8-9). Longitudinal section of inflorescence confirms arrangement of flowers as panicle. As well, secretory ducts in the vascular bundles are seen (Figure A7).

Examining microscopic sections showed that the anther development includes three pre-meiotic, meiosis and post-meiotic steps. In pre-meiotic step, microsporangium wall and spore-forming tissue are produced from division and differentiation of one or more epidermal anthers cell(s). Spore-forming tissue is detected by dividing high colorable cells and dense cytoplasm (Figure B 1). The distance of two ventral sporangia is more than the two dorsal sporangia; at this stage, microsporangia located in a theca are far away from each other. But they are also separated from each other by septum in depth. Along with the spore-forming tissue divisions, tangential divisions of some cells obtained from epidermal cell division are performed to form the anthers walls. Pre-meiotic stage is completed by stopping mitosis of spore-forming tissue and transformation of spore-forming cells to microspore mother cells that are large cells with bulk nucleus and dense cytoplasm (Figure B2). Meiotic stage begins with taking away microspore mother cells from tapetum cells. At this stage, central indentation of each anther theca was completely deep and reaches to the central vascular bundle adjacent of anther, so that the two microsporangia in the theca are completely separated from each other and the septum cannot be seen (Figure B2). Simultaneously with the meiosis I, pectocellulosic wall of microspore mother cells is hydrolyzed and replaced by new callus walls. These walls keep away microspore mother cells from interacting on each other during meiosis.At the time of microspore mother cell meiosis, anther wall is composed of epidermis, a mechanical layer, more than three middle layers and nutritional layer (FigureB6).In plants under salinity treatment, although florets appeared normal, but the growth of the anthers was not normal, and more anthers were small, shriveled in the right of figure B1- 4. Cytoplasm division during meiosis of these cells occurs simultaneously. Inside of each microsporangium at this stage, microspore mother cells with two and four cores are present respectively after meiosis I and II (FigureB7). After meiosis, the cytoplasm division is done by the establishing grooves of the microspore mother cell and these cells are orientated as tetrahedral type and called tetrahedral tetrad. All tetrads are tetrahedral type and four cells are placed in a

common callus wall and also are separated by a callus (Figure B8). Post-meiotic stage begins after breaking the callus wall and passing from tetrad stage to free microspore stage; at the beginning of this process, microspore is still in tetrad arrangement. Microspores have a certain nucleus with marginal position and dense cytoplasm after being released from tetrad at the beginning of differentiation. Then the callus cover disappears completely and the anther wall has mechanical layer, three middle layers and nutritional layer (Figure C1). Finally, middle and tapetum layers disappear with the differentiation of mature pollen grains; tapetum is glandular (Figure C2). Then the effects of thickening of mechanical layer walls become apparent, except the epidermis wall that leads to display of this U- shaped pattern layer. Mature anther wall has the mechanical layer with U- shaped pattern along with the traces of tapetum layer and the middle layer. Disconnection between the two middle walls of each sac with the central part of anther leads to dehiscence in length and depth (Figure C1-3,5). anther was shriveled and pollen grains had non-natural shape(Figure C5-7).

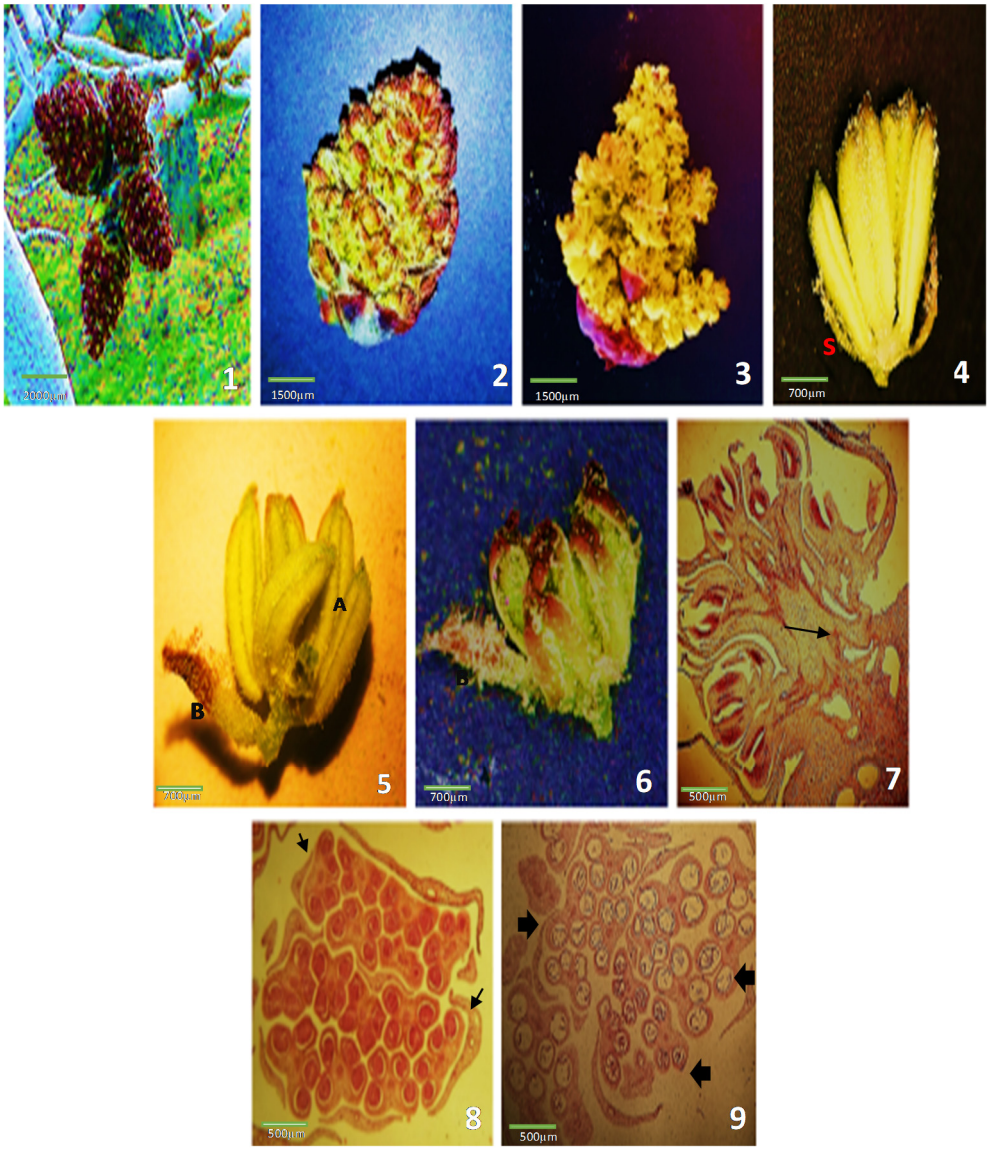

Figure A (1-9)- Morphological and anatomical structure of inflorescence and male pistachio flowers (P. vera): 1- emerging male inflorescence laterally before the leaves; 2- dorsal surface of the male inflorescence bridge along with red anthers; 3- surface of male inflorescence showing to color changes of anthers to yellow; 4-5 male flower panicles with a short peduncle and large anthers whith 2 sepal 1 bract;6 - flower with of dehiscent anther, 7- longitudinal section of inflorescence, the arrow refers to the secretory ducts in the side of vascular bundles; 8-9 cross-section of inflorescence, narrow arrow points flower with five anthers, thick arrow shows flower with four anthers and five anther arrow head shows flower with six anthers and seven anthers, A = Anther, S = Sepal, B = Bract

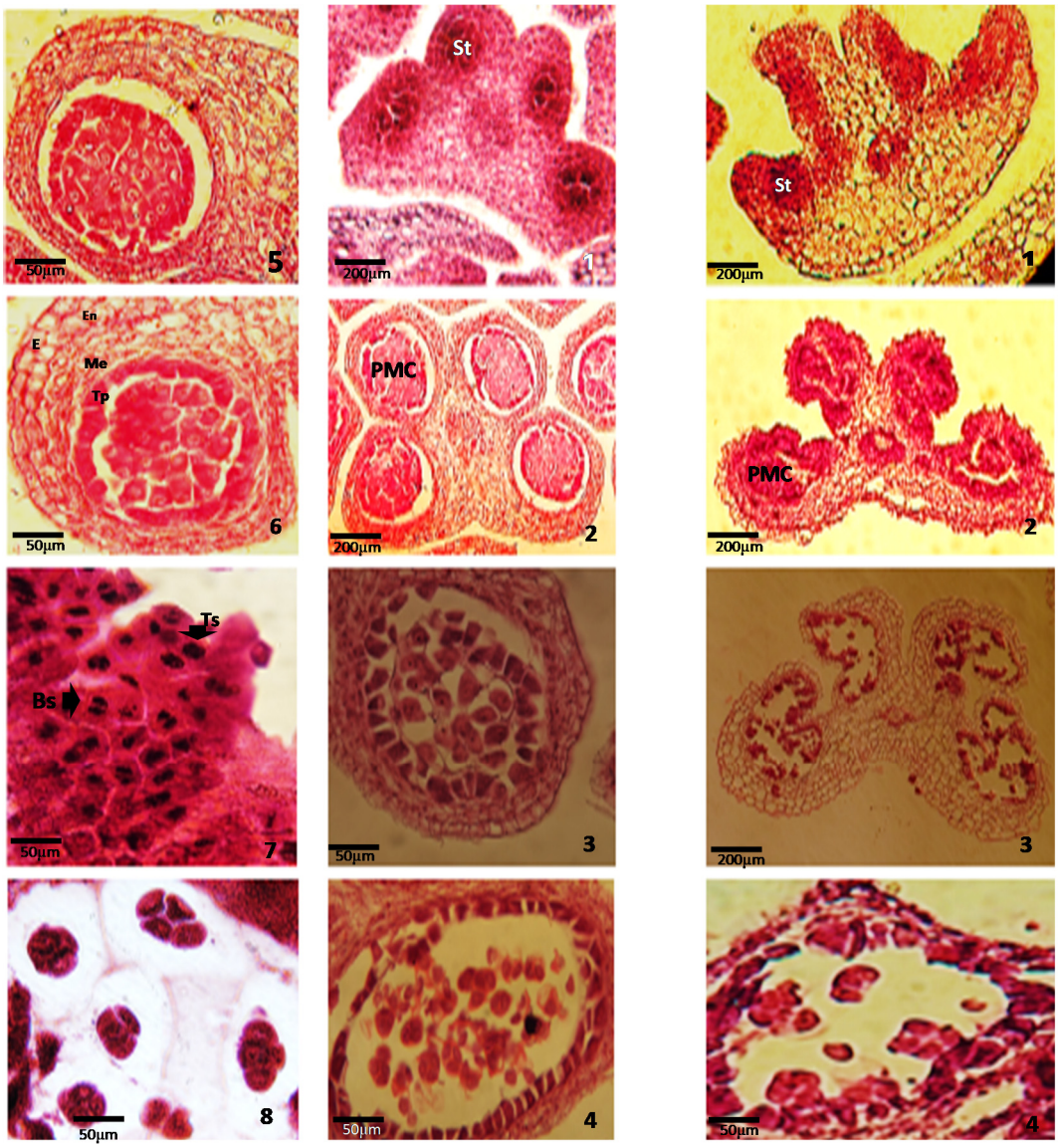

Figure B(1-8)- Stages of forming tetrad microspores inside of anthers in pistachio (*P. vera*) and in plants under NaCl treatment on the right figure: 1- entire cross-section view of anther at the mitosis division of spore-forming tissue cells phase, in plants under salinity treatment on the right figure anthers were small, shriveled; 2- stopping of mitosis in spore-forming tissue cells and microspore mother cells, in plants under salinity treatment on the right figure anthers were small, shriveled and distancing from nutritional layer ; 5- spacing between nutritional layer and microspore mother cells with high magnification; 3 - On the right figure entire view of anthers with microspores mother cells in meiosis and on the left; 6- anther wall of microspore mother cell meiosis time, which is composed of the epidermis, a mechanical layer, more than three middle layers and taptom; 7- PMC in two and four- core stage, 4,8- cytoplasm division simultaneously in the microspore mother cells and production of tetrahedral tetrad surrounded by a thick layer of transparent callus, ST = spore-forming tissue, PMC = microspore mother cells, Ts = quad-core microspore mother cells (tetrad cell), Bs = a dual-core microspore mother cells (dyad cell), TP = nutritional layer

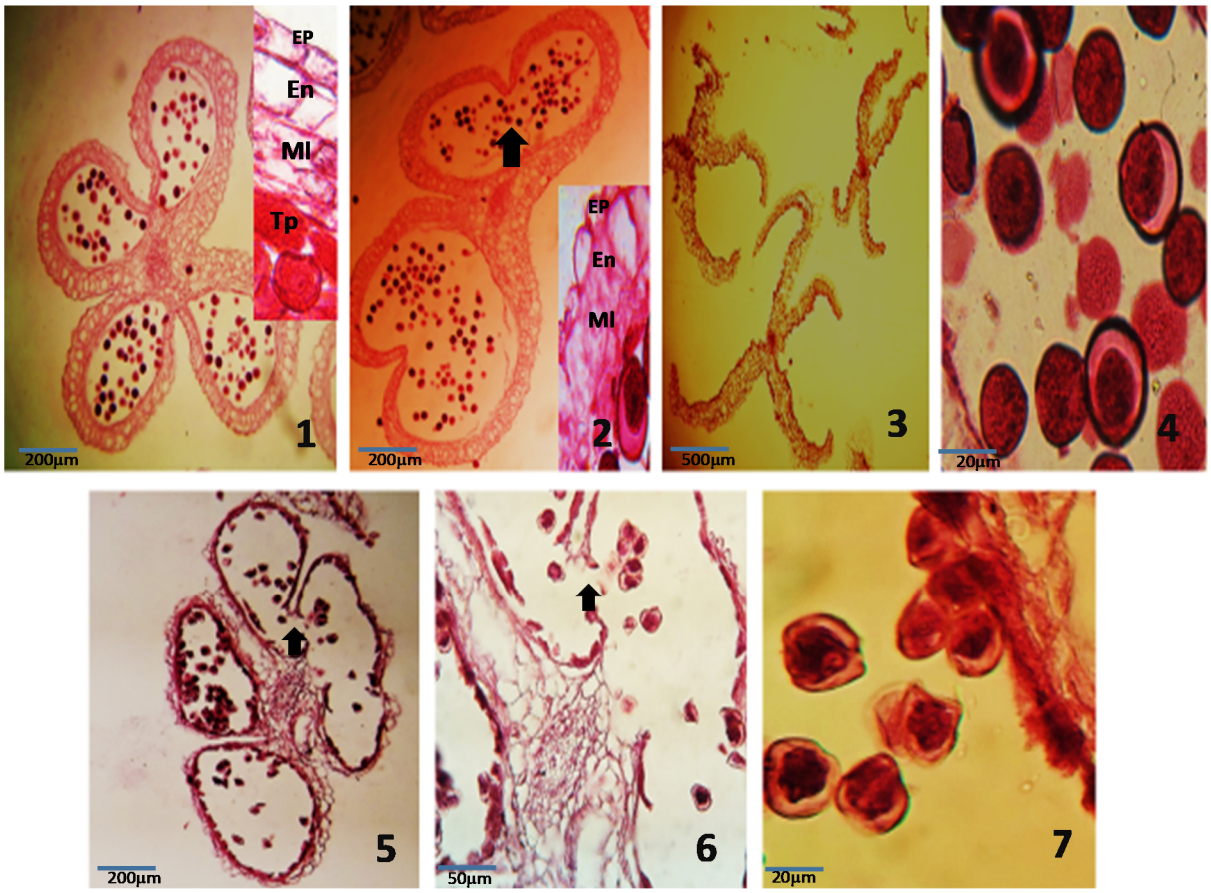

Figure C (1-4)- Anatomical structure of anther cross-sections of pistachio (*P. vera*) and (5-7) in plants under salinity treatment since the production of free microspore to flourish anther: 1- complete decomposition of callus wall surrounding the tetrad and production of free microspores entire cross-section view of anther in the mature pollen Arrowhead points out to break down the walls of a cavity for anther dehiscence. Anthers wall is composed of an epidermis layer, a mechanical wall layer, three middle layers and a tapetum layer, and entir view of anther cross-section at free microspores stage and disappearance of nutritional layer; 2- microspores feeding nutrition layer, leading disappearance of nutritional layers with the middle layer cells,; 3- entire view of dehiscent anther; 4- mature pollen; 5- entire cross-section view of anther in the mature pollen grains. Arrowhead points out to break down the walls of a cavity for anther dehiscence in plants under salinity treatment, 6 anther with mature pollen Arrowhead points out to break down the walls of a cavity for anther dehiscence in plants under salinity treatment, 7- mature pollen under salinity treatment; EP = epidermis, En = mechanical layers, ML = middle layer

4. Discussion and Conclusion

Pistachio is an economically valuable plant that is native to Iran, and for as much as cultivation, it is extensively grown in different areas daily. It is very important to study the bioecological and ecological problems of the plant, including the types of stress such as the NaCl tension in Iran, which is one of the major problems in cities and areas where pistachios are cultivated. Biological stress of pistacia was studied by Seydi et al. (2015), Parsa and Karimian (1975), Ranjbar et al. (2002), Chelli-Chaabouni et al. (2010), and Bastam et al. (2013). Al-Saghir (2010) in the study of genus *Pistacia* L. indicated that flowers are small, monoecious, without petal and at panicle type of inflorescences. This study also showed that flowers are monoecious and are created from female pedicel in complex panicle and on separate pedicels, as well as have 4-6 stamens, 2 or 3 sepals and the 0-1 bract. The number of stamens in the genus Pistacia has been reported 4-6 pcs by Hormaza and Polito (1996), 3-5 pcs by Shiyan (2001), and 4-5 pcs in species *P. chinensis* by Zeng-Fang et al. (2010). Al-saghir (2010) in the study of genus Pistacia L. used bracteole term for non-bracteal excrescences surrounding flowers, as well as male and female flowers of this genus have 1-3 small bracts and 2-7 bracteoles. In addition, Endress (2007) in a study of several species of Pistacia L. introduced non-bracteal excrescences as sepals and reported its numbers in male flowers of *P. lenticus* same as

their stamens numbers, which was 4-6. These researchers stated that they doubt if these excrescences are sepals or bract, they also reported 5-10 sepals-like organs in *P. terbinthus* and only two of them in *P. mexicana*. Moreover, since the flowers of the genus pistacia are pollinating by wind, so it shows inclination of evolution route towards being dioecious and losing perianth (lack of petals and decreased sepals). In our review studies, very few reports were found about developmental stages of anther to produce pollen in the genus pistachios. it individually was studied In the *P. vera* species, by Xu-Xin et al. (2011). In the present study, the anther wall during meiosis of microspore mother cell is composed of the epidermis, a mechanical layer with more than three middle layers and a glandular tapetum layer. The stability feature of middle layers is significant in this species. Because in most plants, middle layers disappear at early stages and before the development of the tapetum layer (Sanders 1999), but in this species, middle layers disappear within tapetum layer at the pollen grains differentiation, Xu-Xin et al. in 2011 have also showed this characteristic. Cytoplasmic division was synchronous after meiosis of microspore mother cell, similar to polygonum type; this finding and glandular tapetum layer are along with results of Xu-Xin et al. (2011) on *P. vera* and results of Zeng-Fang et al. (2010) on *P. chinensis*. In this study, it was observed that cytoplasmic division after meiosis is performed simultaneously by establishing grooves from the around of microspore mother cell toward the center of cell and forming four microspores. These cells are orientated tetrahedrally. All of observed tetrads were tetrahedral. Xu-Xin et al. (2011) found that tetrad of *P. vera* is isobilateral, so there are both types of tetrad in varieties of *P. vera*. Zeng-Fang et al. (2010) reported that *P. chinensis* has both types of tetrahedral and isobilateral tetrads in. In pre-meiotic stage, parts of microsporangia located in one theca are separated from each other via the septum. But in meiotic stage, middle indentation of each theca of anther gets completely deep until it reaches to central vascular bundle of anthers, so that the two microsporangia in a theca are completely separated from each other. This property is also seen in few plants. In most cases, the septum completely separates two pollen sacs until the end of the maturation. Finally, meanwhile its decomposition, two sacs are connected and release pollens through superficial dehiscence pore. The post-meiotic stage begins with decomposition of callus wall and passing tetrad stage to microspore stage. Finally, middle layers and tapetum layer disappear with differentiation of mature pollens. Mature anther wall has the mechanical layer with U- shaped pattern along with the traces of tapetum layer and the middle layer. Disconnection between the two middle walls of each sac with the central part of anther leads to dehiscence in length and depth. The pollens are released cross deep cleavage sites.To convert eggs to grain, the related tissues and cells pass the developmental and physiological processes. Because many food sources is necessary for plant reproduction. Plants set development of pollen grains, eggs and grains in response to changing environmental conditions. In extreme environmental conditions, development of pollen grains will fail at the growing time.In this research, the male gametophyte development steps in pistachio plant were investigated using conventional methods and plant response NaCl stress at this step was studied. The results obtained in this study is in a good agreement with the findings of previous studies on the deterrent effect of salinity stress on male gametophyte development in plants (Namuco OS, O'Toole 1986, Moss 1971, O'Toole 1981, Saini 1981, Sheoran 1996, Westgate 1986). O'Toole, Namuco (1986) showed microspores are sensitive to salinity stress at the growth step. In some crops, including beans, canola, corn and soybeans, stress conditions cause the death of plant cells in the mature gametophytes (Kokubun 2001, Moss 1971, Sage 1990, Young 2004). Therefore, high level ofenvironmental conditions can stop or fail the normal development of gametophyte, embryos and pollen in plants, but this failure in the developmental stages depends on the stage where stress is applied. Male reproductive development in plants is very sensitive to salinity stress and dehydration in PMC during meiosis. During this phase, the water shortage prevents the development of most of microspores or pollen grains and causes male sterility. These injuries apparently are not caused by a direct impact on reproductive tissues and are caused by indirect effect of water shortages in different organs, such as leaves. The mechanism of this reaction may include a remote molecular signal in organs that are under stress and affects fertility in reproductive tissues. A lot of research show involvement of abscisic acid in this relation, but more evidence is required to prove the role of this hormone in the induction of male sterility (McRae 1985, Morgan 1980). Stopping the male gametophyte development induced by stress conditions is resulted by disorders of carbohydrates metabolism, their distribution within the anther and inhibition of the sugar hydrolysis key enzyme, which is invertase. the gene expression of invertase can be regulated by the glucose level. Reduction in the delivery of sugar to the reproductive tissues resulted by reducing photosynthesis by salinity may be a signal, which causes these metabolic changes and results of the failure of the male gametophyte development (Dorion et al.1996, Saini et al. 1984)). Stopping the male gametophyte development that leads to sterility of pollen grains, is the most common among cereals. Drought caused by the salinity stress during the formation of the stamen to the formation of anthers, causes a sharp decline in crops in many dicotyledonous plants (Dubetz & Bole, 1973; Fischer, 1973; Salter & Goode, 1967; Sato, 1954; Turner, 1993; Wells & Dubetz, 1966; Westgate & Peterson, 1993). Abnormalities in the growth of anthers exposed to stress

salinity and water shortage during meiosis were studied by Saini et al. (1984) and Lalonde et al. (1997). They showed microspore stem cells apparently do meiosis completely, but the growth of more microspores is stopped in next steps. The most common symptom of the failure of growth is shifting of microspores from their natural margin position. This can happen at any time between the young microspore step and the first mitosis of the pollen grain. This time apparently depends on the cultivar. In some the anthers, abnormal vacuolation of tapetal layer after meiosis can be observed. Therefore, the incorrect function of tapetal may lead to incorrect orientation of microspores. Studies of Saini, Aspinal (1981) and Sheoran, Saini (1996) showed that pollen grains with inappropriate orientation and diluted cytoplasm has thin antin or without it, but they have exine and do not able to accumulate starch that is the main factor of productivity of the grass pollen grains. The discovery of abnormalities in pairing and separation of chromosomes during meiosis in pollen mother cells in the barley under the salinity and drought stress was studied by Skazkin, Zavadskaya (1957). These abnormalities were introduced as the cause of male sterility. O'Toole, Namuco (1986) also reported that some chromosomal abnormalities in rice are increased under the drought stress. Generally, the findings of this study revealed that the salinity conditions in the flowering step reduce the number of pollen grains in anther. Moreover, anthers growth is not normal under salinity conditions, and some of anthers are wrinkled, discolored, and small. The early destruction of the anther wall, shrinking of pollen grains, and formation of pollen with abnormal shapes and properties prove that the salinity stress reduces the yield of Canola through affecting the male gametophyte development of this plant. Studying separate environmental conditions in the future and changes that occur during the developmental stages of the pistachio growth and reproductive organs is recommended.

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