



RESEARCH ARTICLE

The Biology of Pomegranate Pollen: All about Formation, Morphology, Viability, Germination and Events relating to Sperm Nuclei

Hakan Engin  • Zeliha Gökbayrak* 

Çanakkale Onsekiz Mart University, Faculty of Agriculture, Department of Horticulture, Çanakkale/Turkey

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ABSTRACT

Investigation of pollen biology (i.e. morphology, viability, germination capacity, development of pollen tube and sperm nuclei) of pomegranates, an andromonoecious species, was aimed in this study with the aid of microscopy. Pollens were collected from the *Punica granatum* L. cv. 'Caner II' at different phenological stages. Morphological features showed that the pollen is prolate with smooth exine surface. Viability was higher in the staminate flowers but germination capacity was better in the bisexuals. Pollen germination begins after it leaves the microsporangium. During pollen tube elongation, the pollen cytoplasm, vegetative nucleus and generative cells are transported within the pollen tube. Before entering the pollen tube, the generative cell undergoes mitosis and form two haploid generative cells. Differences in pollen viability and germination ratio of the flower types were found to be insignificant. Polar length was maximum (28.5 µm) in both sexual morphs and minimum (26.8 µm) in the perfect flower. The width of pollen grains ranged from 15.9 µm to 17.1 µm in both types. Perfect and functional male flowers had small pollen (15.9-27.3 µm) with grooves on the surface without perforations. The surface displayed regular continuities where it was between the protrusions. The surface of pollen grains from both of flowers was striate, with more parallel longitudinal ridges in functional male flower. The pollen from both sexes is about the same size. These findings not only provide information on basic features of pomegranate pollen and its pollination biology, but also can help understand breeding and decide strategies to develop better cultivars.

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Introduction

One of the first domesticated fruit species is pomegranate and its cultivation is widespread between subtropical and tropical areas in the world. Although the number of varieties known to the human is around 500, it is said that only 10% is commonly grown (Hancock, 2004). Turkey is not only one of the pomegranate producing countries following Iran, India and Spain, but also has wide selections of germplasm located in different parts of the country.

It is only rational to assume that over the thousands of years of its domestication, pomegranates have gathered many genetical changes in their genome, allowing the species to spread to and successfully grow in different ecological conditions. Pollens in these terms have significant contribution to its genetic makeup. Due to the fact that they are genetically preserved and not under the influence of environmental conditions (Shangshang et al., 2015), their biology has been scientifically investigated in pomegranates (Yang et al., 2013)

* Corresponding author

E-mail address: zgokbayrak@comu.edu.tr

as well as in other plant species. Variations observed in morphology in terms of shape, size and surface characteristics using microscopy present a tool for taxonomic classification (Maiti et al., 2016).

Literature regarding pomegranate pollen studies mainly involve determining pollen morphology (Erdtman, 1971; Zhao and Xiao, 1996; Yin et al., 2011), pollen tube growth (Gadze et al., 2011), in vitro germination capability (Derin and Eti, 2001; Engin and Hepaksoy, 2003, Engin and Gökbayrak, 2017), in vitro germination with different hormones (Engin and Gökbayrak, 2015; Engin and Gökbayrak, 2016), in situ viability (Gökbayrak and Engin, 2018). Yet, no study has ever been done to fully and completely explore pollen's structure, viability, germination capacity, pollen tube development and the events relating to germ nuclei in the anthers. This research aimed to accomplish this goal using microscopy (i.e. light, stereo zoom and scanning electron) in one of the pomegranate cultivar bred in Turkey through selection.

Materials and Methods

Laboratory Analyses

Pollens obtained from the flowers of pomegranate (*Punica granatum* L.) cv. 'Caner II' were used as the material for this study. The plants have been grown at the experimental field of Çanakkale Onsekiz Mart University's Horticulture Farm, 5 m above sea level. The trees were 13 years old, planted at 3x5 m spacing. Since this research was to plan to serve four different purposes (pollen formation, pollen size and shape, viability and in vitro germination, and events relating to the germ cell inside a pollen), every step towards accomplishing them was explained separately.

Pollen formation was determined in the flowers buds at different stages every week from the beginning of April 2018 to the beginning of flowering (May 20, 2018) at 9.00-11.00 am from healthy plants. To avoid physiological deterioration, the flower buds were immediately stored in a polystyrene box with ice. Carnoy's solution (absolute ethanol and acetic acid at 4:1) was used to fix buds for 48 h, then transferred to 70% (v/v) ethanol before putting in a refrigerator. Anthers were slowly detached from the flowers using a forceps and arrow headed needles under Olympus SZX7 stereo zoom microscope (Olympus Corp., Japan). Later, they were fixed in FAA (37% formaldehyde, 70% ethanol, 98% acetic acid in a ratio of 10:80:10 v/v) for 24 h and then preserved in 70% ethanol. Anthers were then washed three times with distilled water and dehydrated in a graded ethanol series (75, 85 and 96%). Samples were prepared for light microscopy using standard methods of the squash technique in acetocarmine (1%) (Johansen, 1940). Stained samples were examined under light microscopy (Olympus CX-41). Microphotographs were photographed using a microscope camera (LC20, Olympus Corp., Japan) mountable on the microscope.

Flowers before the open petal stage were collected to assess pollen size and shape. Forceps were used to detach anthers. Moisture in the anthers were removed by keeping them for about 12-18 hours at room temperature (22°C), which

enabled them to split and release pollen. After following further dehydration of pollens for another 10-12 hours under the same conditions, they were placed in brown glass vials and stored in a refrigerator at +4°C until examined. After preparation of the pollens, the method described by Engin and Unal (2007) for scanning electron microscopy (SEM, Jeol JSM-7100F, Tokyo, Japan) was used with thirty pollen grains from both perfect and functional male flowers. Morphological aspects examined were shape, length of the polar axis (*P*), length of the equatorial diameter (*E*) and ratio of polar axis to equatorial axis (*P/E*).

A colorimetric test of 2,3,5-triphenyl tetrazolium chloride (TTC, 1%) were utilized to determine the viability of the pollens derived from the flowers collected at the open petal stage. The separation of the pollens as viable or not viable was performed based on color tonality (darker meant viable) under a light microscope after a waiting period of two hours. Ratio calculations (%) were made with viable pollens divided to total pollen number. Additionally, the pollen grains were sawn over the medium (20% sucrose and 1% agar) at 26±1°C under 8 hours dark and 16 hours daylight conditions to assess the in vitro germination ability. Twenty-four hours later, germinated and not germinated pollens were counted using a light microscopy (Olympus CX-41) at 10x magnification from a random selection of six-field views and the ratio of germination (%) was calculated.

SEM Analysis

Pollen grains to observe pollen tube development were cultured in a medium containing 20% sucrose in a petri dish. They were kept at room temperature. After waiting for five, ten and twenty hours, samples were put into brown glass bottles, fixed with ethanol (98% purity) and stored in a refrigerator until examined. Before SEM examination, the samples were further dried at room temperature for 4-6 h. For the SEM study, samples were mounted directly on metallic stubs using double-sided adhesive tape and coated with gold in a sputtering chamber (Bal-Tec SCD 005 Sputter Coater). Observation of the prepared samples was carried out with a scanning electron microscope (SEM) Jeol JSM-7100F (Tokyo, Japan) at 15 kV.

Statistical Analysis

The first analysis was performed on the data obtained relating the shape (*P*, *E* and *P/E* ratio), which were from the 10 pollens in 3 replicates. The second analysis was on the viability and in vitro germination ratios of the pollens, which were realized on 6 glass slides. There were minimum 100 pollens counted in each slide. The statistical analysis was performed using MINITAB statistical package software (Minitab Inc., ver.16), and the significant means were compared using Tukey's test.

Results and Discussion

In pomegranate (*Punica granatum* L.) at the beginning of its development, the anther is composed of a mass of cells that appear undifferentiated. As the anther develops, groups of sporogenous cells form. Through meiotic division of these

cells, some of them grow into nutritive cells that supply nutrition for the microspores. However, some produce mother cell (Figure 1, A). Tetrads are typically arranged as the four cells in one plane. After the formation of the four cells, a thick layer of callose, which is darkly stained, coats whole tetrads and separates the individual microspores (Figure 1, B). All tetrads in the anthers within the same size flower bud appear to be at the same developmental stage. After the formation of the four microspores, the development of the pollen grain walls begins. The callose wall is broken down and the free pollen grains are grown in size and develop their characteristic shape and form (Figure 2, A). The wall of pollen displayed two different layers, as exine and intine. The exine is often variously sculptured, and the character of the markings is of value for identifying genus, species, or cultivar. The exine, thin in nature, displays regular continuities (Figure 2, B).

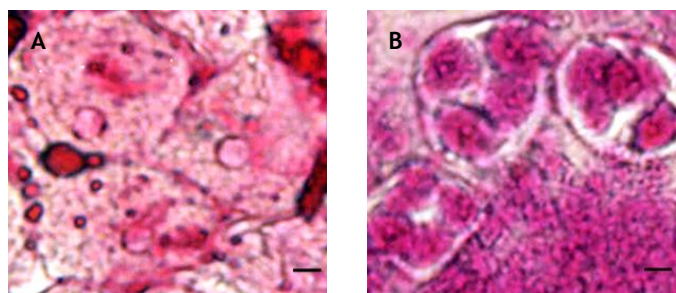


Figure 1. Light microscopy images (Stained with acetocarmine Bars = 20 μm). **A:** Pollen mother cells; **B:** Tetrad showing four microspores separated by callose wall

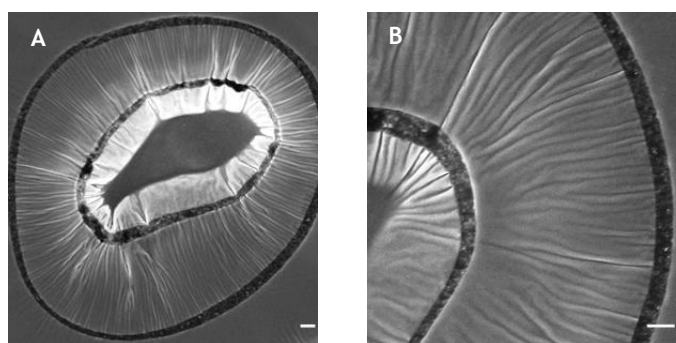


Figure 2. Scanning electron microscopy images (Bars = 1 μm). **A:** Pollen grain; **B:** Mature pollen grain showing thick exine and intine

In 'Caner II' pomegranate cultivar, the formation of pollen occurred very close to the flowering. The first pollen mother cells (PMC) were seen in flower buds which were taken on May 2. The first detection date of tetrads was May 5 and the samples of young pollen grains were also observed. The time

(day) between formation of PMC, tetrads, and pollen grains and the beginning of flowering is given in Table 1.

'Caner II' had two types of flowers on the same tree: hermaphroditic and functional staminate flowers. Pollen from both perfect and male flower types is similar based on the analysis conducted by SEM (Figure 3). Pollen grains have a spheroidal shape with a smooth exine surface. The grains are considered prolate in view of the number, position and type of the apertures.

Table 1. Timing (days before flowering) of the formation of the first pollen mother cells (PMC), tetrads and pollen grains in the pomegranate cultivar

Cultivar	Days before flowering		
	PMC	Tetrads	Young pollen grains
Caner II	23	19	19

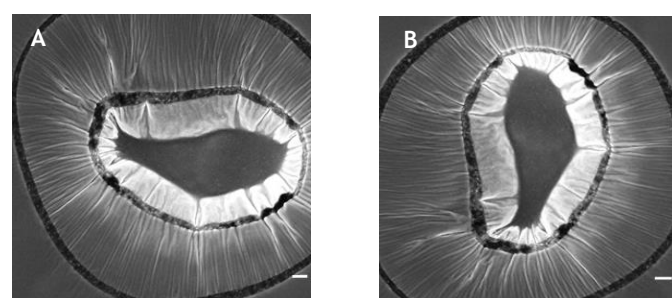


Figure 3. Scanning electron microscopy images (Bars = 1 μm). **A:** Equatorial view of pollen grain of pomegranate cultivar 'Caner II' from functional male flower; **B:** Polar view of pollen grain of pomegranate cultivar 'Caner II' from perfect flower

The values pertaining to the morphological features of the pollens were given in Table 2. The investigated pollen grains in perfect and functional male flowers of 'Caner II' cultivar did not vary in size and shape. Polar length was maximum (28.5 μm) in both sexual morphs and minimum (26.8 μm) in the perfect flower. The width of pollen grains ranged from 15.9 μm to 17.1 μm in both types. The pollen from both sexes is about the same size. According to the classification of Erdtman (1969), the pollen grain of 'Caner II' was prolate. This is in accordance with the findings of Varasteh and Arzani (2009) who characterized the shape of the pollen grains of 14 Iranian pomegranate cultivars as prolate based on *P/E* ratio. *P/E* ratio of 55 indigenous pomegranate cultivars was reported between 1.54 and 2.05 by Shangshang et al. (2015). Engin and Gökbayrak (2017) classified pollen of cultivar 'Caner I', another selection from 'Caner' group, also as prolate.

Table 2. Morphological characteristics of pollen grains from functional male and perfect flowers of pomegranate (*Punica granatum* L.) cultivar 'Caner II' (Mean \pm SE)

	Polar axis (P) μm		Equatorial axis (E) μm		P/E ratio	shape
	Variation range	Mean value	Variation range	Mean value		
Functional male	26.8-27.9	27.35 \pm 0.09	16.1-16.9	16.70 \pm 1.29	1.64 \pm 0.06	prolate
Perfect	26.8-28.5	27.34 \pm 1.01	15.9-17.1	17.06 \pm 1.03	1.60 \pm 0.08	prolate
Pollen (Mean)		27.35		16.89		

Pollen grains obtained from the flowers just before anthesis for scanning electron microscopy examinations show that pollen grains are spherical, and they were approximately 22 μm in size (Figure 3, A). Perfect and functional male flowers of 'Caner II' cultivar had small pollen (15.9-27.3 μm) with grooves on the surface. There were no perforations on the surface of pollen grains. The surface displayed regular continuities where it was between the protrusions. The surface of pollen grains from both of flowers was striate, with more parallel longitudinal ridges from functional male flower. The ridges were more parallel in functional male flowers and less parallel in perfect (Figure 4).

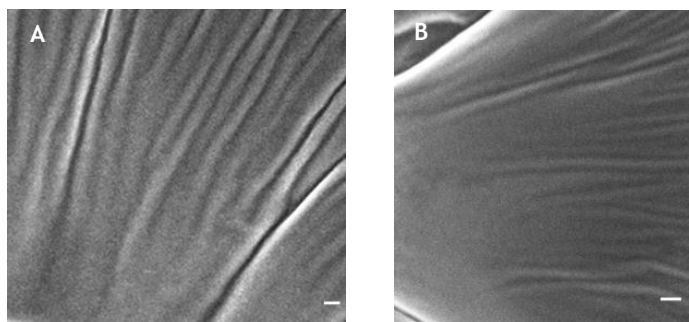


Figure 4. Scanning electron microscopy images (Bars = 1 μm). **A:** Pollen grain surface of from functional male flower; **B:** Pollen grain surface of from perfect flower

Pollen viability and in vitro germination of flowers with different sexes were tested on pomegranate variety 'Caner II' (Table 3) using the TTC test and the results did not exhibit any statistically significant differences, although staminate flowers had slightly higher viable pollens. TTC produced a distinctly clear contrast between viable and nonviable pollen grains (Figure 5). Viability of pollens considerably lower than those in other genotypes reported by Derin and Eti (2001) and Sangma and Singh (2017). Notwithstanding, germination was markedly low in both functional male and perfect flowers and there was not a significant difference in the functional male flowers compared to the perfect flowers. In contrast to the findings of Derin and Eti (2001) and in agreement with Gözlekçi and Kaynak (2000), that the two types of flowers did not produce pollens with significant differences in germination.

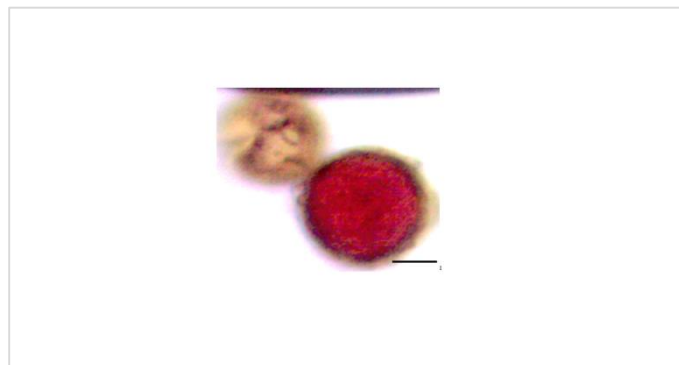


Figure 5. Non-viable (left) and viable (right) pollen grain stained with TTC using light microscopy (Bar = 10 μm)

SEM analysis of pollen grain from perfect and functional male flowers in the pomegranate 'Caner II' cultivar showed that pollen germination begins after it leaves the microsporangium (Figure 6) But in some flowering plants, this might happen before it departs the microsporangium (Carol et al., 2001). During pollen tube formation, a defined area in the pollen plasma membrane promotes a directional growth (Figure 6). Following formation, the longitudinal growth takes place very quickly (Figure 7).

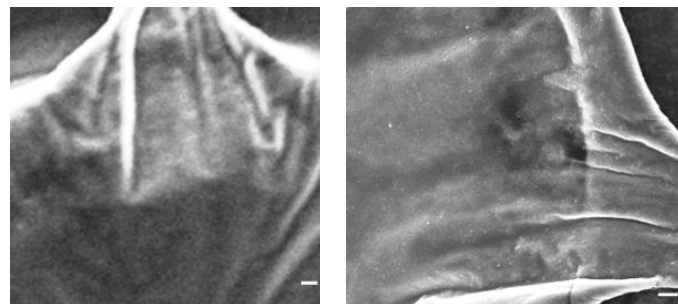


Figure 6. Scanning electron microscopy images (Bars = 1 μm) of pollen tube formation

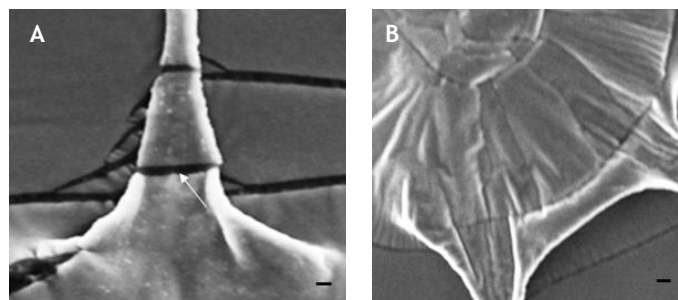


Figure 7. Scanning electron microscopy images (Bars = 1 μm). **A:** Pollen tube elongation (arrow = callose plug); **B:** Two pollen tube elongations from a pollen grain

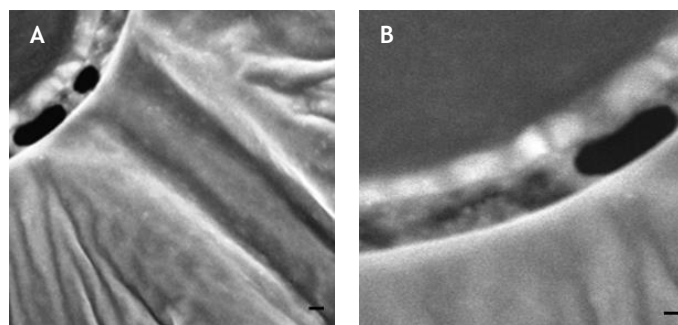


Figure 8. Scanning electron microscopy images (Bars = 1 μm). **A:** Appearance of vegetative and elongated generative cells; **B:** Close-up view of generative cell, going through a mitosis to form two generative nuclei

Barnabas and Fridvalszky (1984) reported pollen tube growth in maize at 1 cm per hour. It is generally accepted that one pollen tube grows out of a pollen but rarely two defined areas in the pollen plasma membrane initiate a directional growth of pollen tubes (Figure 7, B).

Table 3. Comparison of pollen sources (perfect and functional male flowers) in the pomegranate cultivar ‘Caner II’ for pollen viability and *in vitro* germination ratio (%)

Cultivar	Pollen viability (%)			Pollen germination (%)		
	Perfect	Functional male	<i>p</i> value	Perfect	Functional male	<i>p</i> value
Caner II	57.07	62.91	0.314	39.22	20.46	0.089

During pollen tube elongation, the pollen cytoplasm, vegetative nucleus and generative cells are transported within the pollen tube (Figure 8, A). In pomegranate before entering the pollen tube, the generative cell undergoes mitosis and form two haploid generative cells (Figure 8, B). The vegetative nucleus was approximately 4 µm in size (Figure 9, A). The generative nuclei were approximately 3 µm in size (Figure 9, B). At the end of the pollen tube, the cells were discharged, vegetative cell at the front and already degenerated due to lack of microphyll (Figure 10).

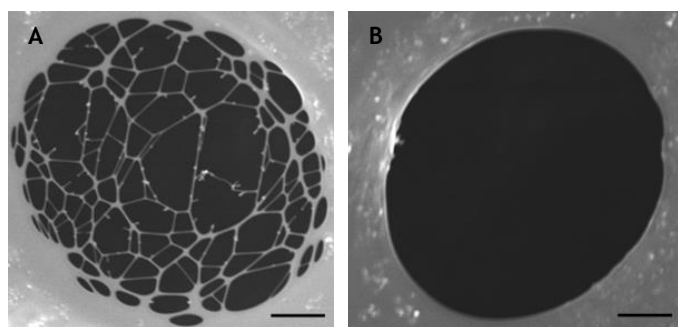


Figure 9. Scanning electron microscopy images (Bars = 1 µm). **A:** Vegetative nucleus at the brink of collapse; **B:** Generative cell

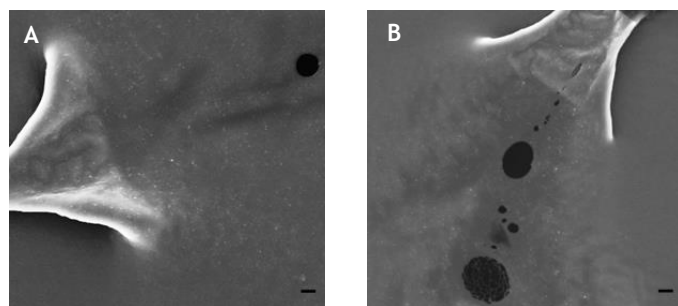


Figure 10. Scanning electron microscopy images (Bars = 1 µm). **A:** Discharge of generative cell out of the enlarged end of pollen tube; **B:** Discharge of both vegetative cell (degenerating) and generative cell from the end of the pollen tube

Conclusion

The information gathered in this study show that In pomegranate (*Punica granatum* L.) at the beginning of its development, the anther is composed of a mass of cells that appear undifferentiated and the formation of pollen occurs very close to the flowering. Scanning electron microscopy examinations show that pollen grains are spherical, approximately 22 µm in size and they have a smooth exine surface. The surface of pollen grains from both of flowers was striate, with more parallel longitudinal ridges in the staminate flower. In view of the number, position and type of the apertures, the grains are prolate. They do not differ in their

size and shape in perfect and functional male flowers of ‘Caner II’ cultivar. Pollen viability is slightly higher in the male flowers. However, *in vitro* pollen germination does not differ with the sexes of the flowers. SEM analysis showed that pollen germination begins after it leaves the microsporangium. Some pollens with two pollen tube growth are also observed.

References

- Barnabas, B., Fridvalszy, L., 1984. Adhesion and germination of differently treated maize pollen grains on the stigma. *Acta Botanica Hungarica* 30: 329-332.
- Carol, A., Rudall, F., Paula, J., 2001. Pollen and anther characters in monocot systematics. *Grana*. 40 (1-2): 17-25. doi:10.1080/00173130152591840.
- Derin, K., Eti, S., 2001. Determination of pollen quality, quantity and effect of cross pollination on the fruit set and quality in the pomegranate. *Turkish Journal of Agriculture and Forestry* 25: 169-173.
- Engin, H., Unal, A., 2007. Examination of flower bud initiation and differentiation in sweet cherry and peach by scanning electron microscope. *Turkish Journal of Agriculture and Forestry* 31: 373-379
- Engin, H., Gökbayrak, Z., 2015. Effect of epibrassinolide, gibberellic acid and naphthalene acetic acid on pollen germination of some pomegranate cultivars. *COMU Journal of Agriculture Faculty* 3(2): 19-25. (in Turkish)
- Engin, H., Gökbayrak, Z., 2016. *In vitro* pollen viability and germination of bisexual and functional male flowers of some Turkish pomegranate cultivars. *Agriculture & Forestry* 62 (4): 91-94.
- Engin, H., Gökbayrak, Z., 2017. Micromorphology of pollen grains from bisexual and functional male flowers of pomegranate. *AGROFOR International Journal* 2(2): 40-46.
- Engin, H., Hepaksoy, S. 2003. Determination of pollen germination of some pomegranate cultivars. *Ege University Faculty of Agriculture Journal* 40 (3): 9-16.
- Erdtman, G., 1969. *An Introduction to the Study of Pollen Grains and Spores*. Copenhagen, Denmark: Munksgaard, pp. 486.
- Erdtman, G., 1971. *Pollen Morphology and Plant Taxonomy: Angiosperms*. Hafner Publishing Company, New York, p.10-18
- Gadže, J., Radunić, M., Petric, I.V., Ercišli, S., 2011. *In vitro* pollen viability germination and pollen tube growth in some pomegranate (*Punica granatum* L.) cultivars from Croatia and Bosnia and Herzegovina. *Acta Scientiarum Polonorum Hortorum Cultus* 10(3): 297-305.
- Gökbayrak, Z., Engin, H., 2018. Effects of foliar-applied brassinosteroid on viability and *in vitro* germination of

- pollen collected from bisexual and functional male flowers of pomegranate, *International Journal of Fruit Science* 18(2): 226-230.
- Gözlekçi, S., Kaynak, L., 2000. Investigations on pollen production and quality in some standards pomegranate (*Punica granatum* L.) cultivars. *Options* No: 42: 71-78.
- Hancock, J.F., 2004. *Plant Evolution and The Origin of Crop Species*. 2nd ed. CABI Publishing, Cambridge, MA, USA.
- Maiti, R., Gonzalez-Rodriguez, H., Ojha, E.R., 2016. Pollen biology and plant productivity: A review. In: Maiti R, Gonzalez Rodriguez H, Sergeevna Ivanova N. (editors). *Autoecology and Ecophysiology of Woody Shrubs and Trees: Concepts and Applications*. 1st ed., John Wiley & Sons, Ltd.
- Sangma, D., Singh, D., 2017. Pollen viability and germination studies of pomegranates cultivars and wild germplasm accessions. *International Journal of Agricultural Sciences* 9(9): 3930-3932.
- Shangshang, Y., Zhaohe, Y., Yanlei, Y., Xueqing, Z., Lijuan, F., Lingling, H., Feng, Z., 2015. Pollen morphology of pomegranate (*Punica granatum* L.) from different eco-geographical populations in China. *Acta Horticulturae* 1089: 269-277.
- Varasteh, F., Arzani, K., 2009. Classification of some Iranian pomegranate (*Punica granatum*) cultivars by pollen morphology using scanning electron microscopy. *Horticulture Environment and Biotechnology* 50(1): 24-30.
- Yang, S., Yuan, Z., Li, Y., Li, Q., Yin, Y., Feng, L., Zhao, X., 2013. Biological characteristics of pollen germination of 'Taishanhong' pomegranate. *Scientia Silvae Sinicae* 49: 48-53.
- Yin, Y.L., Yuan, Z.H., Feng, L.J., Zhao, X., Tao, J. 2011. Comparison of pollen ultrastructural morphology among pomegranate (*Punica granatum* L.) cultivars in Shandong province. *Acta Horticulturae Sinica* 38: 955-962.
- Zhao, X.G., Xiao, L., 1996. A study on pollen morphology of *Punicaceae* from China. *Acta Botanica Boreali Occidentalia Sinica* 16: 52-55.