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Araştırma
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Pollen Performance and Morphology of Black Mulberry (*Morus nigra* L.) Genotypes*

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Abstract

Objective: It was aimed to determine the morphology, viability, germination rates, and production quantity of black mulberry (*Morus nigra* L.) pollens, which play an important role in pollination and fertilization.

Materials and Methods: In this research, pollen viability of black mulberry pollens using the TTC method, pollen germination test using the "agar–plate" method, pollen quantity using the Hemocytometric method, pollen sizes and morphologies using light microscope and scanning electron microscope (SEM) were examined.

Results: In this study, pollen performance and morphological structures of two different dioic male black mulberry genotypes (Genotype 28 and Genotype 5) were investigated. Number of pollen grains per anther was determined as 51666 in Genotype 28 and 54666 in Genotype 5. Pollen viability ratios were 90.4% in Genotype 28 and 89.2% in Genotype 5. Pollen germination ratios of Genotype 28 and Genotype 5 were 68.7% and 72.9% respectively. Pollen shape was spherical and surface ornamentation consisted of small coarse spiny structures and irregular reticulate folds. Polar axis length was measured as 22.08 µm in Genotype 5 and 22.24 µm in Genotype 28. Equatorial axis length was measured as 21.09 µm in Genotype 5 and 21.32 µm in Genotype 28. Pollen germination apertures were evenly distributed on pollen surface, number of apertures varied and apertures were porate (spherical) type.

Conclusion: Present findings revealed that black mulberry genotypes produced plenty of pollen grains, about 90% of produced pollen grains were viable and had a high germination ratio. Such findings indicated that a problem will not be encountered in terms of pollen performance in hybridization studies.

Keywords: Germination apertures, Dioic male genotypes, Pollen viability, SEM imaging (scanning electron microscopy)

Karadut Genotiplerinin (*Morus nigra* L.) Polen Performansı ve Morfolojisi

Öz

Amaç: Bu araştırmada, tozlaşma ve döllenmede önemli rol oynayan karadut polenlerinin morfolojisi, canlılığı, çimlenme oranları ve üretim miktarının belirlenmesi amaçlanmıştır.

Materyal ve Yöntem: Bu araştırmada, karadut polenlerinin polen canlılığı TTC yöntemiyle, polen çimlenme testi "agar-plate" yöntemiyle, polen miktarı hemositometrik yöntemle, polen boyutları ve morfolojileri ışık mikroskobu ve taramalı elektron mikroskobu (SEM) kullanılarak incelenmiştir.

Araştırma Bulguları: Çalışmada iki farklı dioik erkek karadut genotipine ait polenlerin performansı ve morfolojik yapısı incelenmiştir (Genotip 5 ve Genotip 28). Bir anterde üretilen polen sayısının Genotip 28'de 51666, Genotip 5'te ise 54666 adet olduğu tespit edilmiştir. Genotip 28 ve Genotip 5'ten alınan polenlerin canlılık oranları sırasıyla %90.4 ve %89.2 bulunmuştur. Yapılan çimlenme testlerinde Genotip 28 polenlerinin %72.9, Genotip 5 polenlerinin ise %68.7 oranında çimlendiği gözlenmiştir. Polen şeklinin küresel, yüzey yapısında küçük kaba dikenli yapılarla birlikte düzensiz ağ şeklinde kıvrımların yer

aldığı yer aldığı görülmüştür. Polar eksen uzunlukları Genotip 5 polenlerinde 22,08 µm, Genotip 28 polenlerinde 22,24 µm; ekvatoral eksen uzunlukları ise Genotip 5 polenlerinde 21,09 µm, Genotip 28 polenlerinde 21,32 µm olarak ölçülmüştür. Polen çimlenme açıklığının (apertür) polen yüzeyine eşit arlıklarla dağıldığı, sayılarının değişken olduğu ve açıklıkların porat (küresel) tipte oldukları saptanmıştır.

Sonuç: Mevcut bulgular, karadut genotiplerinin bol miktarda polen ürettiğini, üretilen polen tanelerinin yaklaşık %90'ının canlı olduğunu ve çimlenme oranının yüksek olduğunu ortaya koydu. Bu bulgular melezleme çalışmalarında polen performansı açısından bir sorunla karşılaşılmayacağını ortaya koymuştur.

Anahtar Kelimeler: Çimlenme açıklıkları, Dioik erkek genotipler, Polen calılıkları, SEM (taramalı electron mikroskobu)

Introduction

Black mulberry (*Morus nigra* L.) is a deciduous tree belonging to the Morus genus in the Moraceae family of the Urticales order. While Freeman (1978) classified mulberries into 12 species, Huo (2002) reported 14, Martin et al., (2002) more than 30 and Datta (2002) 68 mulberry species. Although there are many mulberry species in the world, the most commonly cultivated mulberry species include; black mulberry (*Morus nigra*), white mulberry (*Morus alba*) and red mulberry (*Morus rubra*) (De Candolle 1967). Mulberry plants (Morus spp.) can exhibit monoecious or dioecious floral structures. Black mulberry is typically a dioecious species, with male and female flowers occurring on separate individuals. Pollination primarily occurs through wind, but insect pollination is also observed (Mogili et al., 2023). Features like germination rates and pollen viability directly impact the success of hybridization studies (Wani et al., 2010). Furthermore, self-incompatibility mechanisms play a crucial role in maintaining genetic diversity (Liu et al., 2022). Several mulberry species grown in different climates in many parts of the world indicate quite a high adaptation capacity of these species (Zhang et al., 1998). With such a good adaptation capability, mulberries are widespread over a wide range of geographies in tropical, subtropical and temperate regions of Asia, Europe, North and South America (Özgen et al. 2009). In India and China, mulberry is grown for leaves as food source of silkworms, while in Türkiye and most European countries, it is generally grown for its fruit. Among the mulberry species, black mulberry is prominent with delicious fruits, high phenolic compounds and pharmacological effects (Anşin and Özkan 1993; Gökmen, 1973). Previous studies on mulberry mostly focused on nutritional values and chemical composition of the fruit. It was indicated in these studies that black mulberry fruits were rich in vitamins and minerals (Khalid et al., 2011; Ahlawat et al., 2017), phenols, anthocyanins, and antioxidants (Özgen et al., 2009). Besides fresh consumption, delicious black mulberry fruits are used for many different purposes such as table fruit, molasses, dried fruit, etc (İslam et al., 2004; Yılmaz et al., 2012).

Türkiye has an important black mulberry population with black mulberry trees growing naturally in different regions. Studies carried out so far on this population have not gone beyond selection studies to determine superior individuals in terms of fruit characteristics (Polat, 2004; İslam et al., 2006; Keskin and Kaya, 2020). To get maximum benefit from the existing genetic richness, it is necessary to initiate large-targeted breeding programs in which the other breeding methods such as crossbreeding or hybridization will be used as well as selection studies. For such breeding programs to be initiated, first of all, pollination biology of mulberries should be well-known. Pollen viability and performance designate the level of success in hybridization studies. Present literature reviews revealed that there was no study on the performance of black mulberry pollens. Therefore, The study of pollen morphology is crucial not only for plant classification but also for plant breeding. Characteristics such as pollen shape, surface structure, and germination apertures influence the compatibility with specific pollination mechanisms, playing a critical role in the success of hybridization studies (Wani et al., 2010; Pandey, 2022). Furthermore, pollen traits provide fundamental data for assessing genetic diversity and selecting superior individuals (Liu et al., 2022). In this context, examining the pollen performance and morphology of dioecious species like black mulberry offers valuable insights for hybridization programs and the conservation of genetic resources (Mogili et al., 2023).

Material and Methods

Pollens were taken from two different previously selected dioic male black mulberry genotypes (Genotype 28 and Genotype 5). The dioecious male

black mulberry genotypes (Genotype 5 and Genotype 28) used in this study were selected from natural populations in and around the Tokat province in northeastern Turkey. The selection criteria included high pollen production capacity, healthy growth conditions, and representative traits for genetic diversity. The trees are 15-20 years old and were chosen for their high pollen viability and germination rates, which make them ideal candidates for this study. Additionally, these genotypes offer significant potential in black mulberry hybridization programs due to their superior pollen performance.

Male catkins were collected before they spread pollen, that is, at the balloon stage, and were instantly brought to the laboratory. Flowers in balloon stage were kept at room temperature for 24 hours to spread pollen. Then, the viability and germination rate of pollens and number of pollens per anther were determined.

Pollen viability: Pollen viability rates were determined using the TTC (2,3,5-Triphenyl Tetrazolium Chloride) method. For this purpose, a 1% TTC solution was prepared, and the pollen grains were incubated in this solution at room temperature for 2 hours. After staining, the pollen grains were examined under a light microscope. Pollen grains stained red were evaluated as viable, those stained light red as semi-viable, and those that remained colorless as non-viable (Figure 1a). This method was previously described by Shivanna and Rangaswamy (1992) and Dafni et al. (2005).

Pollen germination test: The petri agar method was used to determine the pollen germination ability of the genotypes. Germination media were prepared with 1% agar containing 15% sucrose ($C_{12}H_{22}O_{11}$) concentration (Beyhan and Serdar, 2009). Approximately 10–40 ml of the germination medium was poured into petri dishes with a diameter of 8–10 cm, ensuring a height of 2 mm. Once the medium cooled, pollen was sown. Test sieves and soft watercolor brushes were used for homogeneous sowing. After sowing, the petri dishes were covered and incubated in a dark environment at 25°C for 24 hours. After incubation, the petri dishes were stored in a refrigerator during the observation period. Under a light microscope, germinated and non-germinated pollen grains were counted, and germination rates were calculated as percentages. Pollen grains forming a pollen tube longer than their own diameter were considered germinated (Figure 1b).

Pollen quantity: Hemocytometric method was used to determine pollen quantity of black mulberry genotypes. It was performed in three replicates. Fifty unexploded anthers were taken and placed into small glass bottles and kept at room temperature until the anthers burst and pollens released. Then, 5 ml distilled water was added to each bottle. Anthers were kept in water for 2-3 hours and crushed and mixed with the aid of a glass stirrer to get a homogeneous distribution of pollen grains in the water. By blowing into this prepared suspension with a Pasteur pipette, pollen grains were mixed thoroughly once again and a part of suspension taken with the aid of Pasteur pipette was dropped onto the counting chamber of hemocytometric slide. The top of the droplet was covered with a thick special slide and number of pollen grains in 1 mm^3 volume on hemocytometric slide was counted (Figure 1c).

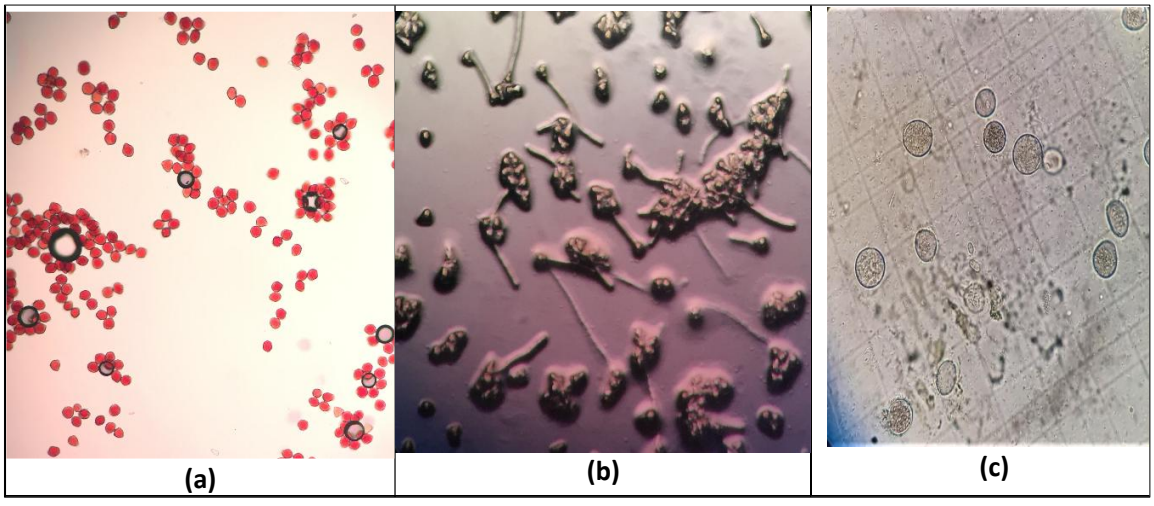

Figure 1. The images related to pollen performance include: a) pollen viability, b) pollen germination, and c) the appearance of pollen on the hemocytometric slide

The Olympus CX23 Binocular Research Microscope was used to determine pollen performance.

Pollen dimensions and morphology: Pollen morphology of two different mulberry genotypes (Genotype 5 and Genotype 28) was examined using light microscope and scanning electron microscope (SEM). Pollen polar and equatorial lengths were measured under a light microscope and general examinations were made. For pollen preparations, anthers were taken on a glycerin dropped slide and crashed with the aid of a stirrer for release of pollen grains, anther residues were cleaned and the slice was covered with a lamella. It was then imaged under light microscope with the use of DP20 digital system and polar and equatorial axis lengths were measured.

Scanning electron microscope (SEM) was used to examine pollen surface structures. Anthers, which were closed at the time of flowering, were fixed in 70% alcohol. At the time of examination, anthers were dried by passing through a series of graded alcohol (70%, 80%, 96% and 99% alcohol) and pollen grains were obtained. Pollen samples were placed on metal carriers with carbon glue under a stereo microscope with the aid of a thin sable brush. Then, the metal carriers were coated with a palladium-gold mixture in a gold-plating device (BAL-TEC SCD005) to make pollen samples conductive and to get a good image under electron microscope. Preparations were examined under ZEISS EVO-40 brand scanning electron microscope (SEM). Suitable regions were scanned.

Results and Discussions

There were an average of 60 anthers in each catkin of Genotype 28 and 88 anthers in each catkin of Genotype 5. Number of pollen grains per anther was 51666 in Genotype 28 and 54666 in Genotype 5 (Table 1).

Table 1. Average pollen production of black mulberry genotypes

Genotype	Number of anthers per catkin	Number of pollen grains per anther	Number of pollen grains per catkin
Genotype 28	60±15	51666±6064.5	3.099.960±910.125
Genotype 5	88±17	54666±2905.9	4.810.608±1.135.577

*Values are presented in mean ± standard error

Although there is no information about number of pollen grains of black mulberry in the literature, Erdogan (2015) reported number of pollen grains per anther of white mulberry as between 1468 - 3134, while Subba Reddi and Reddi (1986) reported the number of pollen grains per anther of the same species as 23388. These values are quite lower than the present values of black mulberry. Subba Reddi and Reddi (1986) worked on 82 wind-pollinated plants of different genera and species and reported that there was a great variability in the number of pollen grains depending on the genera and even the species within the genus and number of pollen grains produced in an anther could reach up to 89.000 in some species. When evaluated on the basis of catkin, it was determined that Genotype 28 produced 3.099.960 and Genotype 5 produced 4.810.608 pollen grains in one catkin.

Besides number of pollen grains, pollen viability also play an important role in successful pollination (Tikader and Dandin, 2007). It was determined that both black mulberry genotypes had a high rate of viable pollen. About 90.4% of the pollen taken from Genotype 28 and 89.2% of the pollen taken from Genotype 5 were viable and the difference was not significant (Table 2).

Table 2. Pollen viability and gemination ratios of dioic-male genotypes

Genotype	Viable pollen ratio (%)	Semi-viable pollen ratio (%)	Germination ratio (%)
Genotype 28	90.4±4.0	6.3±2.9	72.9±6.4
Genotype 5	89.2±2.7	9.3±3.9	68.7±2.1
$LSD_{0.05}$	13.3	5.8	18.7

*Values are presented in mean ± standard error

These values are similar with the viability rates reported for *M. indica* and *M. serrata* species (Tikader and Dandin, 2007). Erdoğan (2015) reported viability rates of white mulberry pollen grains as between 68.81 - 76.62%. Pollen germination ratios were also investigated in this study and it was determined that Genotype 28 had an average germination ratio of 72.9% and Genotype 5 had 68.7%. Khan and Perveen (2008) investigated germination rates of white mulberry pollen grains stored under different conditions and reported germination ratio of pollen grains stored at -20, -30 and -60 C for a week as 69%, 71.3% and 73.1%, respectively. These findings revealed that pollen viability and germination rate

were high in black mulberry and the other mulberry species.

Pollen Morphology

Pollen samples of Genotype 5 and Genotype 28 were investigated under light microscope and scanning electron microscope (SEM) and pollen appearances of the genotypes are presented in Figures 2, 3, 4 and 5. Polar axis and equatorial axis lengths of the genotypes were also measured. In Genotype 5, average polar axis length was measured as 22.08 µm and equatorial axis length was measured as 21.09 µm. In Genotype 28, average polar axis length was identified as 22.24 µm and equatorial axis length as 21.32 µm. It was determined that number of germination apertures varied. There are three or four-aperture regions and apertures are of porate (spherical) type (Figures 2, 3). Although rare, there were five-aperture pollen grains (Figure 2.D).

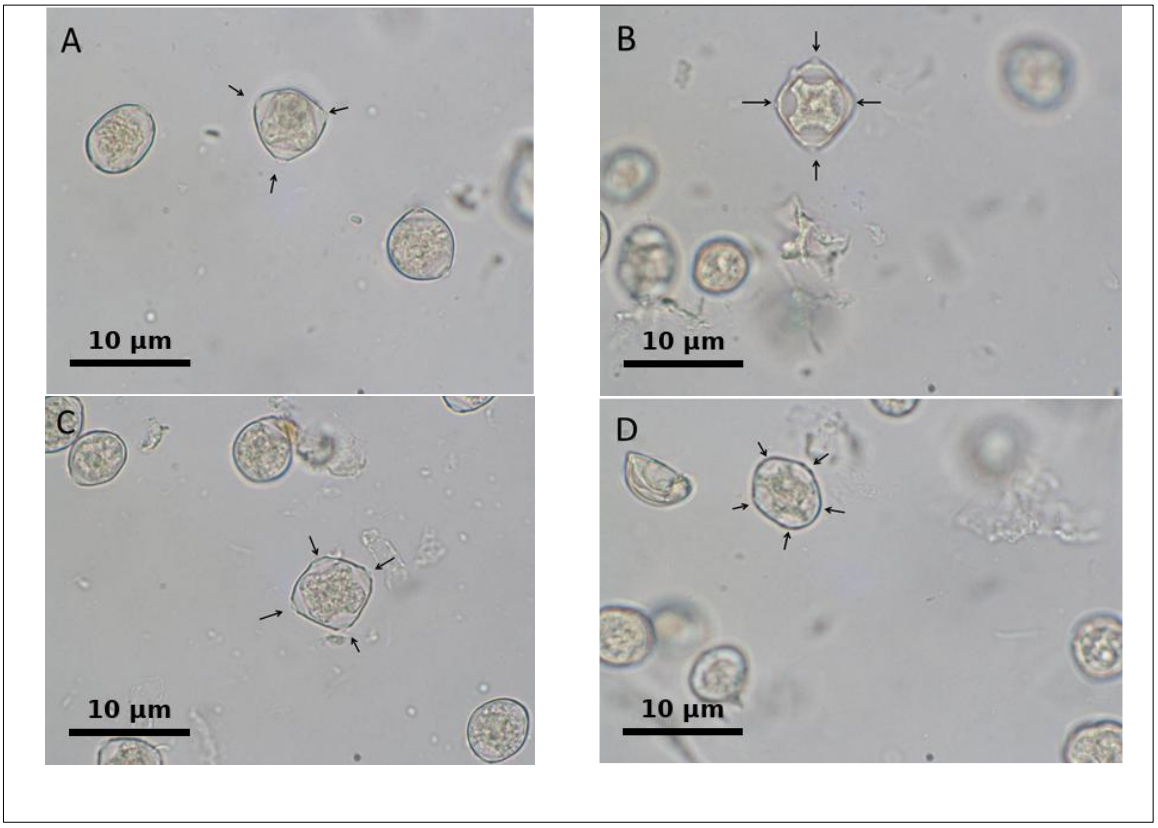

Figure 2. Light microscope image of pollen grains of Genotype 5. A; three-aperture ones, B,C; four-aperture ones D; five-aperture ones. Germination apertures were indicated with arrows

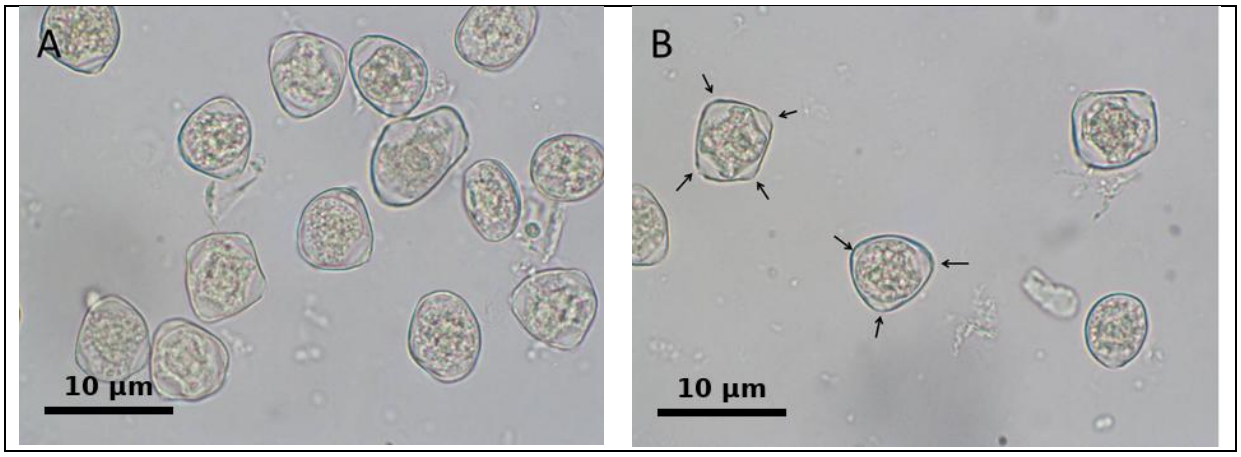

Figure 3. Light microscope image of pollen grains of Genotype 28. A and B; three and four-aperture ones. Germination apertures were indicated with arrows

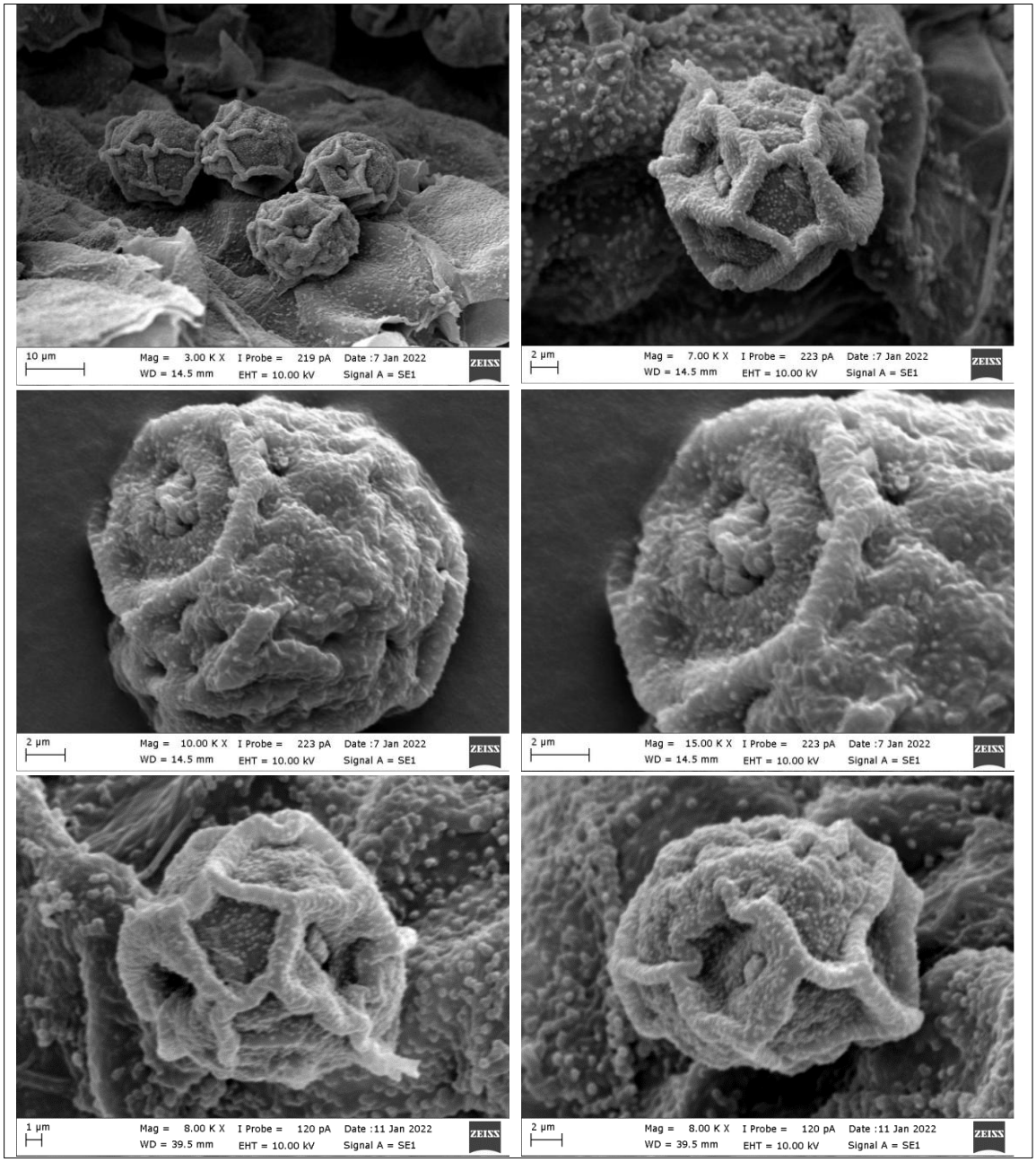

Figure 4. Scanning electron micrographs of pollen grains of Genotype 5

Germination apertures were not evenly spaced on pollen surface. It was reported that *Morus nigra* pollen grains had 3, 4 and 5 germination apertures (Tojyo 1966; Punt and Malotaux 1984). Gelorini and Bourgeois (2005) stated that black mulberry (*Morus nigra*) pollen grains had 3 porate-shaped germination apertures not evenly distributed on pollen surface. Zhenjiang et al., (2015) investigated pollen characteristics of 10 diploid and tetraploid genotypes of *Morus atropurpurea* to provide a scientific basis for polyploidy breeding of mulberry. It was reported that pollen grains were small and round in shape, polar axis lengths varied between 16.98 - 24.46 µm, equatorial axis lengths between 15.37 - 23.86 µm and number of germination apertures between 1-3. Those findings support the present ones. SEM images revealed that pollen shape and pollen ornamentation were the same in both genotypes. Pollen shape is spherical. Pollen surface structure included small coarse spiny structures and irregular reticulate folds. It was seen that there were two ring-shaped structures around the germination apertures and there was a germination aperture right in the middle of these two ring structures.

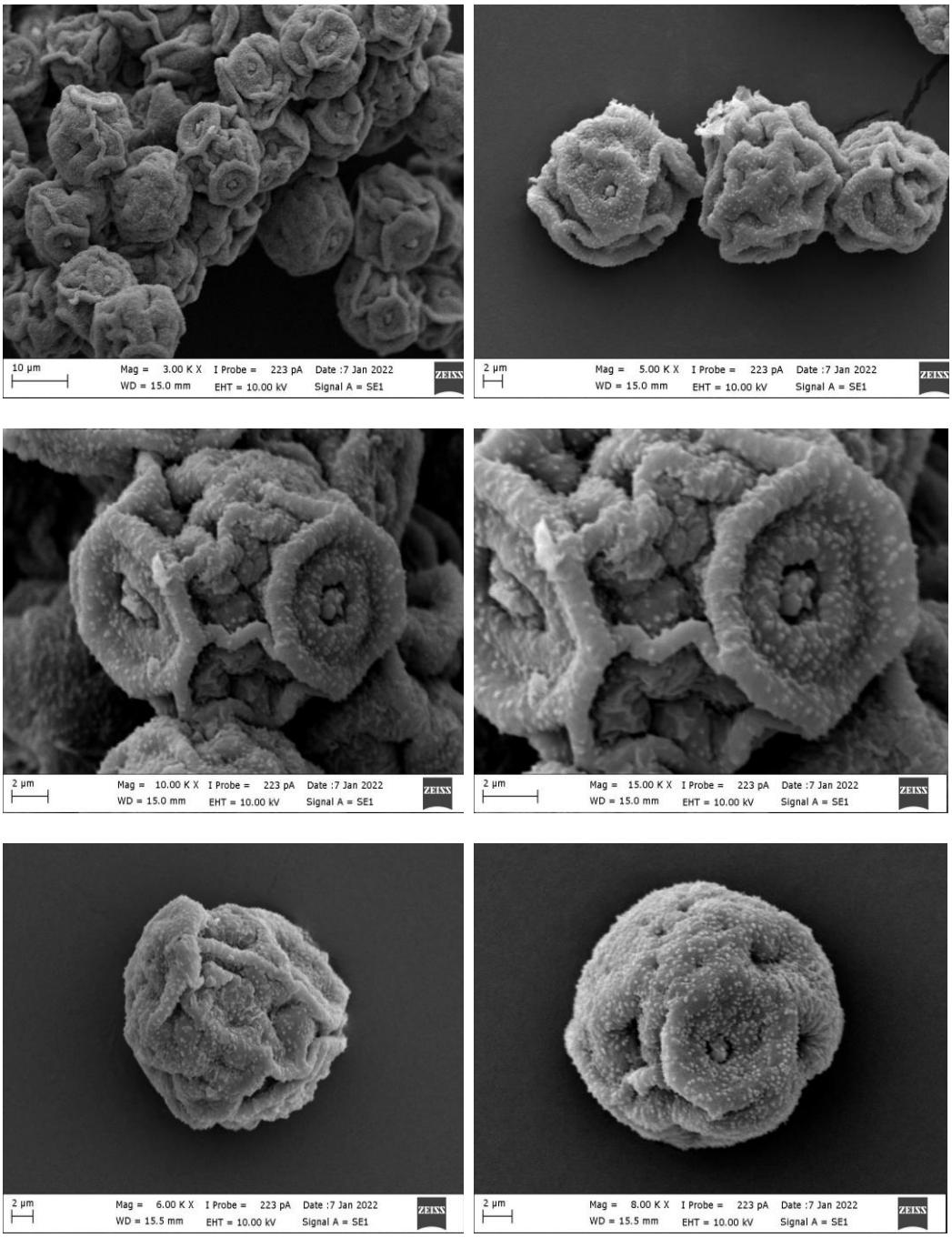

Figure 5. Scanning electron micrographs of pollen grains of Genotype 28

Morus nigra pollen has rarely been described. Punt and Malotaux (1984) indicated that pollen ornamentation of *Morus nigra* was scabrate, that is, there were only coarse spiny structures on pollen surface and these structures were unevenly distributed. Pollen ornamentation of the present genotypes was different from this definition. There may be differences in pollen surface structure of the genotypes within the same species. Indeed, Zhenjiang et al., (2015) investigated pollen characteristics of 10 diploid and tetraploid genotypes of *Morus atropurpurea* and reported differences in pollen size and pollen ornamentation of diploid and tetraploid varieties. These researchers classified pollen grains into four types based on surface ornamentation as: (1) coarse spiny structures and fine cerebroid (brain-like) strips, (2) coarse spines and coarse cerebroid strips, (3) smooth spines and fine cerebroid strips, and (4) smooth spines and coarse cerebroid strips.

Conclusion

Present findings revealed that black mulberry genotypes produced plenty of pollen grains, about 90% of produced pollen grains were viable and had a high germination ratio. Such findings indicated that a problem will not be encountered in terms of pollen performance in hybridization studies.

Pollen morphology is an important tool used in the classification of plants. There is little information available in the literature about the pollen morphology of black mulberry. With the present study, morphology of black mulberry pollens was described in detail.

Conflict of Interest

The authors declare no conflicts of interest.

Authors Contribution

All authors contributed equally.

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