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Artificial Pollination and Fruit formation in Black Mulberries (*Morus nigra* L.)

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ABSTRACT

The purpose of this study was to investigate the pollination and fertilization biology of black mulberry (*Morus nigra* L.), with a specific focus on understanding the effects of different pollination treatments on fruit formation and seed formation. Two experiments were designed to evaluate both dioecious and monoecious genotypes. In the first experiment, genotype 25 (dioecious female) was subjected to various artificial pollination treatments using pollen from two male genotypes (genotype 5 and genotype 28), as well as isolation treatments to observe parthenocarpic fruit formation. High fruit formation rates were recorded across all treatments, and no significant differences in fruit size or drupelet number were observed, regardless of the pollen source. The second experiment involved three monoecious genotypes (genotype 1, genotype 30, and genotype 31), where significant variations in fruit formation and size were observed, depending on the pollen source. This study highlights the potential for both fertilized and parthenocarpic fruit formation in black mulberry and underscores the importance of pollen source in determining fruit quality and seed formation.

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Introduction

Black mulberry (*Morus nigra*) is a species that has spread across temperate and subtropical regions of the northern hemisphere, originating from Asia and the Caucasus, and has adapted well to various ecological conditions. It has long been utilized for its fruit, leaves, roots, and bark, all of which are noted for their medicinal properties (Datta, 2002). In recent years, the economic value of black mulberry fruits has increased due to their use in the food industry (e.g., cakes, confectionery, ice cream) and their rich nutritional content. Despite its increasing commercial importance, research on the pollination and fertilization biology of black mulberry remains scarce.

Research on black mulberry (*Morus nigra*) in Türkiye is not limited to fruit selection studies but spans various disciplines, including the effects of drought stress in vitro (Vijayan et al., 2014), propagation through tissue culture (Švagr et al., 2023), and preservation techniques such as genetic conservation through collection orchards (Uzun & Bayır, 2009). Recent studies have explored the genetic diversity, adaptability to different environmental conditions, and physiological responses of black mulberry under various stress factors, contributing to its agricultural and ecological importance (Gnanesh et al., 2023; Abbas & Rehmat, 2020). These multidisciplinary approaches provide a deeper understanding of Morus nigra and its potential for further use in breeding and conservation programs. In addition to the collection orchards of different mulberry species established in various parts of the world, including India, a mulberry conservation orchard has also been established in Türkiye. Although Tokat hosts a significant population of black mulberry (*Morus nigra*), there are no established black mulberry orchards, and the exact number of trees and genotypes remains unknown. Studies are primarily focused on the preservation and improvement of local mulberry genotypes. Hybridization studies and conservation efforts are being conducted to enhance the genetic diversity, adaptability, and fruit quality of mulberry species (Das & Krishnaswami, 1965; Dwivedi et al., 1989; Tikader & Dandin, 2007).

In order to maximize the benefit from the existing genetic richness in Turkey, it is necessary to initiate large targeted breeding plans in which other breeding methods will be used in addition to selection studies. For this purpose, it is essential to examine the flower structure and fertilization biology of mulberries in detail. Black mulberry flowers are typically unisexual, with male and female flowers found on separate trees (dioecious) or occasionally on the same tree (monoecious) (Gnanesh et al., 2023). The first condition for seed and fruit formation is the formation of healthy male and female flowers and then successful fertilization. For successful fertilization, pollen emitted from the male organ must reach the style of the female flower, where it germinates, develops a pollen tube, and transfers the generative nucleus to the ovary (Janick & Moore, 1996; Thompson, 1996).

In addition to fertilization, black mulberry can also form fruits through parthenocarpy, where fruit develops without fertilization, resulting in seedless fruit. This phenomenon is important for ensuring fruit production under conditions where pollination may be unreliable. Parthenocarpy can be naturally induced or stimulated through specific hormonal changes, such as increased levels of auxin or gibberellin, which promote fruit growth in the absence of seed development (Griggs & Iwakiri, 1973). The occurrence and implications of parthenocarpy in black mulberry were further supported by Gustafson (1942), who extensively discussed natural and artificial parthenocarpy. Parthenocarpy in black mulberry thus provides a potential advantage for stable fruit yields, as it allows fruit to form even when pollinators are scarce.

Understanding parthenocarpy in black mulberry is crucial for improving fruit yield and quality, as well as for developing more efficient breeding programs. Studies in other fruit-bearing species have shown that parthenocarpy can improve fruit set and size, providing a means to mitigate the risks associated with fluctuating pollination conditions (Wilcock & Neiland, 2002; Young & Young, 1992). This study focuses on the reproductive biology of black mulberry, with an emphasis on artificial pollination and fruit formation in monoecious and dioecious genotypes, to enhance agricultural strategies and the breeding potential of black mulberry.

Materials and Methods

In this study, due to the absence of dedicated black mulberry orchards in Tokat, artificial pollination was conducted using black mulberry trees located in individual orchards across the region. The female genotype (genotype 25) used in the experiment was characterized by a larger fruit size and an early harvest date, typically around. The hybridisation study did not alter the harvest date. Phenological observations, including bud burst, first flowering, and end of flowering, are provided in Table 9. The male genotypes (genotypes 5 and 28) were selected based on their floral characteristics, including the number of male flowers per inflorescence, as reported by Demirel and Yıldız (2021). Two different experiments were established for this purpose to study the effects of these genotypes on fruit formation and seed development.

Experiment 1

In the first experiment, we selected one dioecious female tree (genotype 25) and used pollen from two dioecious male trees (genotype 5 and genotype 28) for pollination. The flowers on the dioecious female tree (genotype 25) were pollinated with pollen from these male trees to evaluate the effect of different pollen sources.

In this experiment, a single tree of this genotype was used, and 12 branches facing in different directions were selected from this tree. Three of these branches were randomly selected and pollinated with pollen from genotype 28, which produces only male flowers, and isolated so that no other pollen could be taken from outside. Three branches were pollinated with the pollen taken from genotype 5, which forms only male flowers, and isolated so that they do not receive any other foreign pollen from outside. Three randomly selected branches were left open for open pollination. The remaining three branches were completely covered to observe whether parthenocarpic fruits were formed. The predetermined branches of black mulberries, selected for both parthenocarpy observations and pollination treatments, were isolated with specially made cloth bags according to the branch size, which provide air permeability but do not allow the passage of pollen and other particles

Experiment 2

Three monecious genotypes (genotype 1, genotype 30, genotype 31) were used as main plants. On the trees of these monoecious genotypes (genotype 1, genotype 30, genotype 31) and the male genotypes (genotypes 5 and genotypes 28) used for pollination, 12 branches from each genotype, facing different directions, were selected for the experiments. In each of these genotypes (genotype 1, genotype 30, and genotype 31), the flowers on 3 randomly selected branches were artificially pollinated with pollen taken from genotype 28 and were then isolated using specially designed cloth bags. These bags allowed air permeability but prevented any pollen from entering, ensuring no contamination from outside pollen. Three branches were artificially pollinated with pollen from genotype 5 and isolated so that they would not receive any other pollen from outside. Three branches were left open for open pollination. The remaining three branches were emasculated first and then covered to observe whether parthenocarpic fruits were produced or not. Since the identified trees were monoecious, i.e. male and female flowers were in different places on the same tree, special care was taken to ensure that there were no male flowers in the isolated branches.

Pollination was done twice, two days apart, to ensure that pollen reached all flowers in the inflorescence.

During the fruit ripening period, the number of fruits on each branch was calculated as a percentage of the initial number of flowers on that branch. Additionally, the fruit retention rate was expressed as the percentage of flowers that successfully developed into fruits.

Flowers (a total of 712) that were artificially pollinated with genotype 28 and genotype 5, left for open pollination, and isolated to prevent pollen exposure were examined, of which 667 successfully developed into fruits. This larger sample size ensures a robust and reliable basis for statistical analysis. For each treatment, three branches from each genotype were used as replicates. The branches were selected randomly from different directions on the trees to account for variability. Each branch was considered a replication, and the fruits were sampled randomly from these branches.

- The number of seeds was determined by manually extracting the seeds from each drupelet in the inflorescence. The seeds were then counted individually for each drupelet to ensure precise measurement.
- Number of drupelet (number): Total number of drupelet in a cluster
- Fruit dimensions: Including fruit width (mm), fruit length (mm), and fruit weight (g), were measured using a digital caliper for size measurements and a precision electronic balance for weight measurements.

Statistical Analysis

All data were analyzed using analysis of variance (ANOVA) to determine the significance of differences between treatments. A significance level of $p < 0.05$ was used for all tests. The statistical analysis was performed using **SAS** software.

Results

Experiment 1

Table 1 shows the fruit formation rates obtained from artificial pollination studies where dioecious genotype 25 (producing only female flowers) was used as the main plant. As seen in the table, almost all pollinated flowers in every treatment developed into fruit, and there was no significant difference in fruit formation rates between the different pollen sources.

There was no significant effect of pollination type or pollen sources on fruit size. The average weight of the fruits taken from the branches pollinated with pollen of genotype 5 was 4.0 g, while the average fruit weight was 2.7 g in the branches that were closed to prevent foreign pollen entry (Table 2). The difference between these two values was not statistically significant. Similarly, it was determined that pollination types (pollination with pollen from genotype 5, genotype 28, free pollination, and isolated branches) did not have a significant effect on fruit size measured as fruit length and fruit width. The fruit width was between 15.2 and 17.0 mm, and the fruit length was between 22.6 and 20.4 mm, depending on the treatments. The number of drupelet (nucs) formed from each flower on an inflorescence (between 18.4 and 18.08) was similar in 4 different pollination treatments.

When the number of seeds formed as a result of fertilization was examined, it was determined that 15.3 and 15.4 seeds were formed in the flowers artificially pollinated with the pollen of genotype 5 and genotype 28, respectively. While this number was 4.3 in the flowers left to open pollination, it was determined that there were no seeds in the flowers that were isolated and not allowed to be pollinated. This result shows that black mulberry can form parthenocarpic fruit without fertilization (Table 2).

Experiment 2

The results of different pollination treatments applied to the monecious genotype 1 used as main plant are shown in Table 3. According to this; all of the flowers pollinated with genotype 28 turned into fruits. Out of 43 flowers pollinated with genotype 5, 24 of them turned into fruit and 55.8% fruit formation rate was obtained. Out of 45 flowers left to open pollination, 43 of them turned into fruit. Again, 98.8% of the flowers isolated to prevent flower dusting turned into fruit.

Some characteristics of fruits from monoecious genotype 1, pollinated using different methods (pollination with pollen from genotype 5, genotype 28, free pollination, and isolation to observe parthenocarpy), were showed in Table 4. It was determined that pollination with different genotypes (genotype 5 and genotype 28) caused significant changes in fruit weight. Flowers pollinated with the pollen of genotype 28 formed larger fruits compared to those pollinated with the pollen of genotype 5 and those pollinated with the pollen of genotype 28. The fruit weight in the treatment pollinated with pollen from genotype 5 was 2.33 g, whereas in the treatment pollinated with pollen from genotype 28, the fruit weight increased to 4.35 g. Similar situation was also observed in fruit size. Fruit width and fruit length were higher in fruits formed from flowers pollinated with genotype 28 pollen. The number of drupelets in a fruit varied between 15.4 and 21.3, but the differences were not statistically significant based on an ANOVA test ($p > 0.05$), which was used to compare the effects of different pollination treatments on drupelet number.

The results of different pollination treatments applied to genotype 30 were showed in Table 5. As can be seen from the table, the fruit formation rate of the flowers pollinated with genotype 5, as well as the flowers left for free pollination and completely closed flowers, was over 95%, while the fruit formation rate of the flowers artificially pollinated with the pollen of genotype 28 was 64.3%.

The average fruit weights of the fruits from genotype 30 varied between 3.05 and 3.31 g according to the treatments (Table 6). Fruit width varied between 15.1 mm and 16.7 mm and fruit length between 20.05 and 21.7 mm. There was no statistically significant difference between pollination treatments in terms of both fruit weight and fruit size. When the number of seeds in a fruit was examined, it was determined that no seeds were formed in the covered flowers and the fruits formed were parthenocarp. Fewer seeds (4.3 seeds) were formed in the free pollination treatment compared to those artificially pollinated with the pollen of genotype 5 and genotype 28. In the closed flowers, no seeds were formed, indicating the occurrence of parthenocarpy, where fruit forms without fertilization. The lower seed count in the free pollination treatment may be attributed to lower pollen viability or reduced pollination efficiency. These findings suggest that while black mulberry can form parthenocarpic fruit, successful pollination significantly increases seed production, which in turn positively affects fruit size and quality. Thus, artificial pollination with selected genotypes can be a viable method to enhance fruit characteristics.

Table 1. Fruit formation rates of genotype 25 pollinated in different ways

Pollen Source	Number of Pollinated Flowers	Number of Flowers Turning into Fruit	Fruit formation rate (%)
Genotype 28	58	58	100 a
Genotype 5	51	51	100 a
Open pollination	42	41	97.6 a
Closed	44	43	97.7 a
LSD	ns	ns	ns

The difference between the averages indicated by the same letter is not significant (P<0.05).

Table 2. Effect of pollination method on the fruit characteristics of genotype 25

Pollen Source	Fruit Weight (g)	Fruit Width (mm)	Fruit Length (mm)	Drupelet Number (per fruit)	Seed Number (per fruit)
Genotype 5	4.0±0.5	17.0±0.7	22.6±0.6	18.4±1.6	15.3±0.5a
Genotype 28	4.0±0.8	16.8±1.2	22.5±1.7	19.6±2.3	15.4±2.8 a
Open pollination	3.0±0.2	15.8±0.6	20.4±1.1	18.3±1.6	4.3± 0.1b
Closed	2.7±0.3	15.2±0.7	20.4±0.8	18.5±0.8	0.0±0.0 c
$LSD_{0.05}$	ns	ns	ns	ns	4.2

The difference between the averages indicated by the same letter is not significant (P>0.05)

Table 3. Fruit formation rates of genotype 1 pollinated in different ways

Pollen Source	Number of Pollinated Flowers	Number of Flowers Turning into Fruit	Fruit formation rate (%)
Genotype 28	43	43	100 a
Genotype 5	43	24	55.8 b
Open Pollination	45	43	95.5 a
Closed	89	88	98.9 a
LSD	ns	ns	ns

The difference between the averages indicated by the same letter is not significant (P>0.05).

Table 4. Effect of pollination method on the fruit characteristics of genotype 1

Pollen Source	Fruit Weight (g)	Fruit Width (mm)	Fruit Length (mm)	Drupelet Number (per fruit)	Seed Number (per fruit)
Genotype 5	2.33±0.57 b	14.1±1.2 b	18.4±2.2 b	15.4±3.7	10.6±4.5 a
Genotype 28	4.35±0.40 a	17.5±0.2 a	24.1±0.9 a	21.3±1.6	17.7±1.7 a
Open pollination	2.74±0.49 b	15.0±0.5 ab	19.1±1.1 b	17.4±1.4	14.5±2.2 a
Closed	2.84±0.40 ab	15.1±0.8 ab	20.7±1.5 ab	17.6±1.9	0.0±0.0 b
$LSD_{0.05}$	1.53	2.56	4.9	7.6	8.5

The difference between the averages indicated by the same letter is not significant (P<0.05).

Table 5. Fruit formation rates of genotype 30 pollinated in different ways

Pollen Source	Number of Pollinated Flowers	Number of Flowers Turning into Fruit	Fruit formation rate (%)
Genotype 28	28	18	64.3 b
Genotype 5	49	47	95.9 a
Open pollination	45	43	95.5 a
Closed	48	46	95.8 a
LSD	ns	ns	ns

The difference between the averages indicated by the same letter is not significant (P<0.05).

Table 6. Effect of pollination mode on fruit characteristics of genotype 30.

Pollen Source	Fruit Weight (g)	Fruit Width (mm)	Fruit Length (mm)	Drupelet Number (per fruit)	Seed Number (per fruit)
Genotype 5	3.13±0.18	16.6±0.3	20.5±0.4	15.9±0.6	12.3± 0.9 a
Genotype 28	3.13±0.42	15.1±2.0	21.0±2.2	18.3±2.2	14.7±2.3 a
Open pollination	3.31±0.24	16.7±0.2	21.7±0.2	16.7±0.5	4.3± 0.4 b
Closed	3.05±0.54	15.9±0.8	21.6±0.7	16.3±0.9	0.0±0.0 c
$LSD_{0.05}$	1.2	2.2	3.8	4.0	4.1

The difference between the averages indicated by the same letter is not significant (P<0.05).

Fruit formation rates of genotype 31 pollinated in different ways are given in Table 7. As seen in the table, 78.3% of the flowers pollinated with pollen from genotype 28 resulted in fruit formation, while all the flowers in the other treatments produced fruit.

The weight of the fruits from genotype 31 was between 2.44 g and 2.88 g, fruit length was 18.4 mm and 20.0 mm, and fruit width was between 14.6 mm and 15.8 mm (Table 8). The effect of pollination type on fruit size was found to be insignificant. In this genotype, the number of drupelet in a fruit varied between 14.2 and 17.5, but the difference was found to be statistically insignificant. When the number of seeds, which is an indicator of successful fertilization, was examined, it was determined that more seeds were formed in artificial pollination treatment with genotype 5 and genotype 28 pollen compared to open pollination. Even though no pollination occurred, fruit still developed in these flowers, but no seeds were formed, indicating the occurrence of parthenocarpy.

Table 7. Fruit formation rates of genotype 31 pollinated in different ways

Pollen Source	Number of Pollinated Flowers	Number of Flowers Turning into Fruit	Fruit formation rate (%)
Genotype 28	23	18	78.32 b
Genotype 5	37	37	100 a
Open pollination	47	47	100 a
Closed	20	20	100 a
LSD	ns	ns	ns

The difference between the averages indicated by the same letter is not significant (P<0.05).

Table 8. Effect of pollination type on fruit characteristics of genotype 31.

Pollen Source	Fruit Weight (g)	Fruit Width (mm)	Fruit Length (mm)	Drupelet Number (per fruit)	Seed Number (per fruit)
Genotype 5	2.88±0.5	15.8±0.9	19.8±1.9	15.3±1.5	13.7±1.6 a
Genotype 28	2.45±0.3	14.7±1.0	19.7±1.3	17.5±1.1	15.0±1.0 a
Open pollination	2.49±0.3	15.3±0.3	20.0±0.6	16.1±0.9	6.5±1.8 b
Closed	2.44±0.2	14.6±0.3	18.4±1.2	14.2±1.4	0.0±0.0 c
$LSD_{0.05}$	1.0	2.37	4.31	4.01	5.21

The difference between the averages indicated by the same letter is not significant (P<0.05).

Table 9. Some phenological observations of the genotypes

Genotype	Sex Expression	Bud Burst	First Flowering	End of Flowering
Genotype 1	Monoecious	13.04.2019	2.05.2019	30.06.2019
Genotype 5	Dioecious -Male	14.04.2019	5.05.2019	2.07.2019
Genotype 25	Dioecious -Female	13.04.2019	7.05.2019	5.07.2019
Genotype 28	Dioecious -Male	12.04.2019	8.05.2019	9.07.2019
Genotype 30	Monoecious	10.04.2020	5.05.2020	6.07.2020
Genotype 31	Monoecious	09.04.2020	8.05.2020	8.07.2020

Discussion and Conclusion

In the experiment where dioecious female genotype 25 was used as the main plant, high fruit formation was observed in all pollination treatments. This result reveals that this genotype does not show incompatibility to foreign pollen and can also form parthenocarpic fruits. Cross-pollination has been reported to be common in black mulberry (Abbas & Rehmat, 2020), as well as in other mulberry species (Gnanesh et al., 2023).

When monecious genotypes were used as main plants, significant differences in fruit formation were observed depending on the pollination method. The flowers of genotype 1 pollinated with pollen from genotype 5 showed lower fruit formation rates compared to the other pollination treatments. While there was a high rate of fruit formation in closed flowers with no pollen (parthenocarpy), the lower fruit formation rate in flowers pollinated with pollen from genotype 5 may be related to excessive pollen load. Although there are no studies on this subject with mulberry species, it has been reported that in some other plant species, excess pollen may prevent fruit formation depending on the incoming pollen source (Wilcock & Neiland, 2002; Young & Young, 1992). In the experiment where genotype 30 and genotype 31 were used as main plants, lower fruit formation was obtained from pollination with genotype 28 pollen. This shows that artificial pollination of mulberries may give different results depending on the pollen source.

The high rate of fruit formation in flowers that were closed to prevent pollen from entering clearly indicates parthenocarpic fruit formation in black mulberry, meaning that these fruits developed without fertilization. The absence of seeds in these fruits indicates the absence of fertilization. Griggs & Iwakiri (1973), in their artificial pollination studies on *Morus rubra*, found that 89.3% of the fruits obtained from flowers pollinated through controlled pollination—where pollen from a specific source is manually applied to the flowers—contained seeds. In contrast, 87.5% of the flowers subjected to free pollination also produced fruits with seeds, while no seeds were formed in flowers that were isolated to prevent exposure to foreign pollen, indicating parthenocarpic fruit formation.

In addition to the positive effects of the varieties used as pollinators on fruit formation, their contribution to fruit quality parameters is also important. This condition, called metaxenia, is one of the main issues to be considered in the selection of suitable pollinators (Jahed, 2015). Metaxenia refers to the phenomenon where the pollen source directly influences the characteristics of the resulting fruit, such as size, weight, and shape, even though the pollen itself does not contribute genetically to the fruit's formation. In this study, the metaxenia effect was observed only in genotype 1, as the weights and sizes of the fruits varied significantly depending on the pollen source. Specifically, fruits pollinated with pollen from genotype 28 were larger and heavier compared to those pollinated with pollen from genotype 5, indicating a clear influence of the pollen source on fruit development.

There was significant variability in the number of flowers within each cluster that successfully fertilized and formed seeds, both in artificial pollination and open pollination conditions. It was thought that this may be due to the fact that not all of the flowers in an inflorescence are capable of fertilization or that the time of receptivity (able to accept pollen) is different.

It was determined that there was no incompatibility problem in terms of fertilization in the black mulberry trees in the population examined in the study, and that they can form fruit as a result of fertilization, but they can also form parthenocarpic fruit without fertilization. In artificial pollination studies, it was determined that the number of drupelets (individual fruitlets within a single fruit) varied significantly depending on the pollination method used. This indicates that different pollination techniques can influence the development of each flower in an inflorescence into individual drupelets, affecting the overall fruit structure and size. Although it was determined that the cluster can turn into fruit even if no flower is pollinated, it was revealed that the number of seeds increases the fruit size.

The findings of this study contribute to the understanding of reproductive biology in black mulberry, providing valuable information for future breeding and cultivation efforts. The identification of parthenocarpy in black mulberry could help inform breeding programs that aim to improve fruit yield, especially in areas where pollination is unreliable. Additionally, the observed metaxenia effect provides new insights into how pollen source can directly influence fruit characteristics, which could be useful for improving fruit quality in commercial cultivation.

Declarations

Author Contribution Statement

*Mehmet Akif DEMIREL:*Conducted the study, data collection, review, formal analysis, and writing the original draft.

Kenan YILDIZ: Management, supervision, review, editing, and statistical analysis of the study.

Conflict of Interest

"We declare that the authors have no conflict of interest."

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