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Rose Pollen Management Methods to Improve Productivity

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Abstract: Roses are one of the most highly produced and purchased ornamental plants worldwide. Procurement and preservation of pollen is essential for the production of diverse rose varieties. In this study, we analyzed pollen management conditions, such as the pollen collection stage, drying time, and storage temperature, to determine optimal conditions for rose pollen management. Pollens were stored under different conditions and the pollen vitality and germination rate were investigated through an optical microscope. The vitality of pollen was an essential factor for rose breeding and depended on the storage conditions. Collecting pollen in the seventh flowering stage resulted in a relatively higher pollen yield. Drying the flower for 5 h after the anther opened improved pollen germination. The germination rate of freshly collected pollen was similar to that of pollen stored at temperatures between −20 °C and −72 °C for up to 30 days, indicating the efficacy of pollen storage at sub-zero temperatures. Since the rate of fruiting increases when pollination is performed three times, considering the time and cost of breeding, it is appropriate to pollinate three times to increase the number of seeds. This study provides an efficient pollen management method to collect and store pollen for breeding.

Keywords: pollen viability; pollen germination; crossbreeding; pollen morphology

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1. Introduction

Rapidly changing environmental conditions greatly impact the functional aspects of plants [1]. Weather anomalies, such as high temperature and drought stress, are becoming major global problems [2], causing growth stress in plants. Therefore, plant breeding methods need to be improved to develop diverse and stress-resistant plants. Newer plant varieties are developed through crossbreeding. For routine crossbreeding of plants, long-term storage of pollen is required, regardless of the flowering time [3]. Effective pollination is a prerequisite for fruit and seed set in most plants, and information on pollen biology, including pollen viability, is important to increase productivity [4–6]. The storage method may affect pollen's viability [7–12]. Pollen is sensitive to temperature and quickly loses viability under natural conditions [13]. Studies have also reported that preservation at room temperatures reduces the viability of pollen rapidly, but storage at low temperatures maintains the viability of pollen for longer periods of time [10]. Appropriate management conditions for each variety should be identified before procuring the pollen for breeding.

Roses belong to the genus *Rosa* of the Rosaceae family and are the most commonly produced and consumed flower species in the world [14]. In addition to their commercial importance as cutting flowers, roses are also important components of gardens, parks, and green spaces. Roses (*Rosa* spp.) have been cultivated for at least 5000 years since the ancient civilizations in China, West Asia, and North Africa [15,16]. They are economically important ornamental plants with characteristics such as a pleasant scent and antioxidant properties [17–20]. In Korea, roses have a cultivation area of 2,470,000 m^2 and account for 9.7% of the total domestic flower production [21]. New rose varieties are developed mainly

using artificial breeding, and securing viable pollen is essential for high-efficiency breeding fruits [22,23].

An important factor with respect to rose breeding is pollen conservation and identifying parameters related to pollen fertility and viability. Pollen conservation affects pollen aging, and preservation is affected by storage conditions [24]. The vitality of rose pollen is preserved for long durations in dry and low-temperature conditions and degraded in conditions of excessive moisture and high temperature. The pollen germination power significantly decreases after 4 to 5 days at temperatures above 25 °C [25]. When the temperature and relative humidity are high during the pollen storage, pollen vitality is greatly reduced in most species [26]. Relative humidity has a greater influence on the change in pollen vitality than the temperature [11,22,27–30]. However, some species such as *Rosmarinus officinalis* have shown excellent pollen vitality under high temperature and high humidity conditions, indicating that temperature and humidity tolerance differ within species [31].

Previous research on rose pollen includes studies on the morphological classification of pollen [23], investigation of physiological mechanisms [4], the establishment of storage conditions for pollen in consideration of cultivation aspects, and enhancement of pollen vitality under unfavorable conditions. However, research on specific rose-pollen management methods to increase breeding efficiency is lacking. Therefore, we conducted this study to find a suitable pollen management method to increase the fruiting rate during artificial crossbreeding of roses and to understand the actual fruiting effect through crossbreeding. We investigated the collection-stage condition of the anther, the drying time, and the storage temperature of the pollen to understand pollen management. The effect of hybridization in terms of the number of pollinations using stored pollen was analyzed.

2. Materials and Methods

2.1. Materials

To analyze pollen storage conditions, five varieties of standard roses ('Green Star', Red Square', 'Shooting Star', 'Mystic Blue', 'Tineke') and five varieties of spray roses ('A-Pink', 'Sunny Lady', 'Ever Spring', 'Vivien', 'Red Pop') were selected as experimental varieties (Figure 1). Flowers that bloomed in May, the natural flowering period after winter, were collected to obtain pollen for the experiment. To investigate the effect of the number of pollinations on fruiting when crossbreeding with stored pollen, experiments were conducted by dividing the roses into a standard breeding combination and a spray breeding combination. The standard combination used the two varieties 'Akito' and 'Calypso' as the female parents and 'Mishell' as the male parent, while the spray hybridization used the two varieties 'Charming' and 'Pink Charm' as the female parents and 'Tineke' as the male parent. The roses used in the experiment were cultivated in five plastic greenhouse buildings of 7 m × 32 m, with an area of 1120 m^2. We regulated the temperature, ventilation, pest control, and fertilizer application as per the rose cultivation method developed by the Rural Development Administration [32].

2.2. Pollen Storage Conditions and Germination Characteristics

2.2.1. Pollen Storage Conditions

To determine the appropriate cut-off harvest time, roses are divided into nine stages of flowering [32]. For our study, we divided five types of standard roses and spray roses into nine stages of growth, from young buds to full bloom (Figure 2). We collected anthers from the flowers during stages 3, 5, and 7 and deposited them in three round, 9 cm Petri dishes, 5 mL each. The Petri dishes were maintained at 26 °C and 60% humidity for five hours. After pollen containers (anthers) were opened, the amount of pollen in the Petri dishes was determined visually. However, the weight of pollen could not be measured because of static electricity in the Petri dish. This pollen was sprayed on the germination medium, and the normal pollen rate and pollen germination rate were determined. To understand the drying time characteristics of the pollen, three samples of anther were collected at the

third stage of flowering from each variety. The samples were stored at room temperature (26 °C) and 60% humidity for 5 h, and for 1, 3, and 7 days, before spraying the pollen on the medium. To investigate the effect of storage temperature on the pollen characteristics, we collected the anther from three varieties and dried them for 8 h. After the drying period, the anthers were stored at four different temperatures (−72, −20, 0, and 26 °C) for 30 days and then studied using the same method.

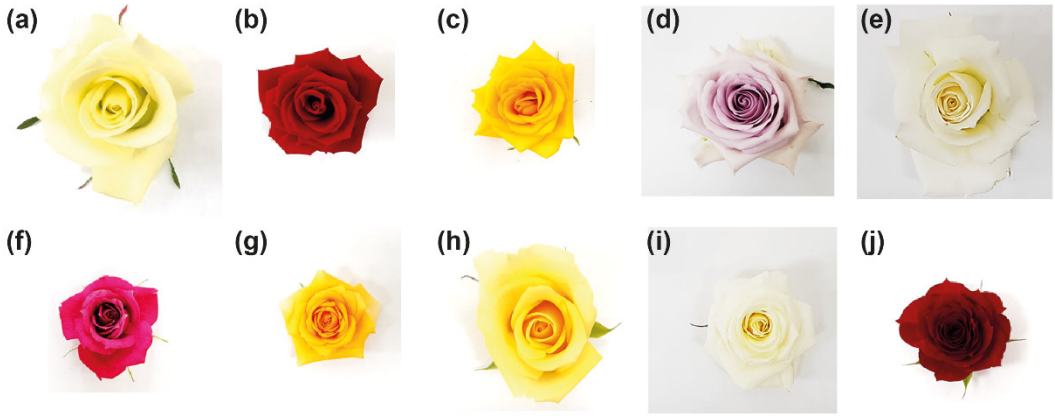

Figure 1. Photos of each variety of roses selected for the experiment. Standard type: (**a**) 'Green Star', (**b**) Red Square', (**c**) 'Shooting Star', (**d**) 'Mystic Blue', (**e**) 'Tineke'; spray type: (**f**) 'A-Pink', (**g**) 'Sunny Lady', (**h**) 'Ever Spring', (**i**) 'Vivien', (**j**) 'Red Pop'.

Figure 2. Flower development stages (1–9) of the 'Shooting Star' rose variety.

2.2.2. Pollen Vitality and Germination Rate

Normal pollen swells to a clear spherical shape, but abnormal (non-viable) pollen maintains the original shrunken, irregular shape [7]. Therefore, pollen with a non-crinkled shape was considered normal (viable). The collected anther was placed in a Petri dish at 26 °C and 60% humidity for 5 h. After this period, the Petri dish was sealed with Parafilm and frozen at −19 °C, and the shape and fertility of the pollen were examined under a microscope. The fluorochromatic reaction (FCR) test was performed to confirm pollen fertility. To perform the FCR test with a confocal microscope (LSM510, Carl Zeiss Co., Jena, Germany), 2 mg of fluorescein diacetate was dissolved in 1 mL of acetone to prepare a stock solution and stored in a freezer. Just before use, two drops of stock solution were dropped into 1 mL of 12.5% sucrose with rose pollen, vortexed using a vortex mixer (VM-01, Korea Ace Scientific, Seoul, Korea) at a speed of 1000 rpm for 20 s, and kept at room temperature. After approximately 5 min, we observed pollen with a confocal microscope (Carl Zeiss LSM510, Jena, Germany) at a wavelength of 488 nm using an LP505 filter. The dark green fluorescence was counted as active pollen and converted into a percentage per total pollen. We repeated the experiment three times per treatment. The prepared pollen was placed in

an isoamyl acetate solution, subjected to critical point drying (HCP2, Hitachi Co., Tokyo, Japan), then coated using an ion sputter coater (E-1010, Hitachi, Co., Tokyo, Japan) and inspected with a scanning electron microscope (S-3000N, Hitach Co., Tokyo, Japan) at an acceleration voltage of 15–18 kV. An optical microscope (Axioskop-2, Carl Zeiss Co., Jena, Germany) was used to observe the pollen by staining it with the periodic acid–Schiff staining method.

For the rose pollen germination experiment, a medium containing sucrose (100 $g \cdot L^{-1}$), agar (5 $g \cdot L^{-1}$), and H_3BO_3 (0.02 $g \cdot L^{-1}$) was used. Pollen was collected on a brush (size: no. 7, diameter: 15.4 mm), applied evenly on the medium to avoid agglomeration, and then germinated at 26 °C for 9 h. The germinated pollen was observed with an optical microscope (Stemi 2000-C, Carl Zeiss, Baden-Württemberg, Germany) and digitally photographed (Cytovision 3.8, Carl Zeiss, Baden-Württemberg, Germany). The pollen germination rate was determined for the germination when the length of the pollen tube was larger than the diameter of the pollen [33]. After three observations under a microscope, the germination rate of the pollen was calculated by dividing the elongation of the pollen tube by the total pollen grains.

2.3. Pollination and Fruiting

To analyze the effect of the number of pollinations when crossbreeding using stored pollen on the fruit, standard crossbreeding and spray crossbreeding combinations were used. Anther was collected during petal opening to investigate the pollen vitality of the male parents, 'Mishell' and 'Tineke'. At 10 am on 4th June, pollen was combed on the stigma head using a brush (no. 7) and pollinated 1, 2, 3, and 5 times, and the fruit set percentage was determined on the 30th, 60th, and 90th days.

2.4. Statistical Analysis

For statistical processing, descriptive statistics for measurement indicators were produced using the software Statistical Package for the Social Sciences (SPSS, version 25.0; IBM SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) analysis was performed to verify the significance of each type and treatment condition. Duncan's multiple range test (DMRT) was performed as a post hoc test to verify the difference between treatments. Pearson's correlation analysis was conducted to understand the correlation between each indicator.

3. Results

3.1. Characteristics of Pollen Vitality in the Flowering Stage

We analyzed the amount of pollen collected in steps 3, 5, and 7 and found that the pollen yield differed for each variety. In most varieties, the amount of pollen was the highest in step 7 (Figure 3). The amount of pollen was related to the number of breeding crosses.

The normal pollen rate of the standard type was 46.4–58.9% at the third stage of flowering and 47.2–58.1% at the fifth stage. The spray-type normal pollen rates were 63.1–84.2%, 62.4–80.6%, and 65.9–83.3%, for stages 3, 5, and 7, respectively (Table 1). The ratio of the normal pollen rate of the spray type was higher than that of standard type. In the standard type, the normal pollen count increased in the following order: 'Green Star', 'Tineke', 'Mystic Blue', 'Red Square', and 'Shooting Star', irrespective of the flowering stage. 'Green Star' and 'Tineke' had higher normal pollen rates as flowering progressed, whereas 'Red Square' and 'Mystic Blue' had higher normal pollen rates at stage 5. 'Shooting Star' had a higher normal pollen rate at stage 3. There was a difference in the tendency of the normal pollen rate according to the flowering stage. In the spray type, the proportion of normal pollen was high in the order 'Ever Spring', 'Red Pop', 'A-Pink', 'Vivien', and 'Sunny Lady'. 'Sunny Lady' at stage 3, 'A-Pink' and 'Vivien' at stage 5, and 'Ever Spring' and 'Red Pop' at stage 7 showed high percentages of normal pollen rates, but there were no significant differences according to the flowering stage.

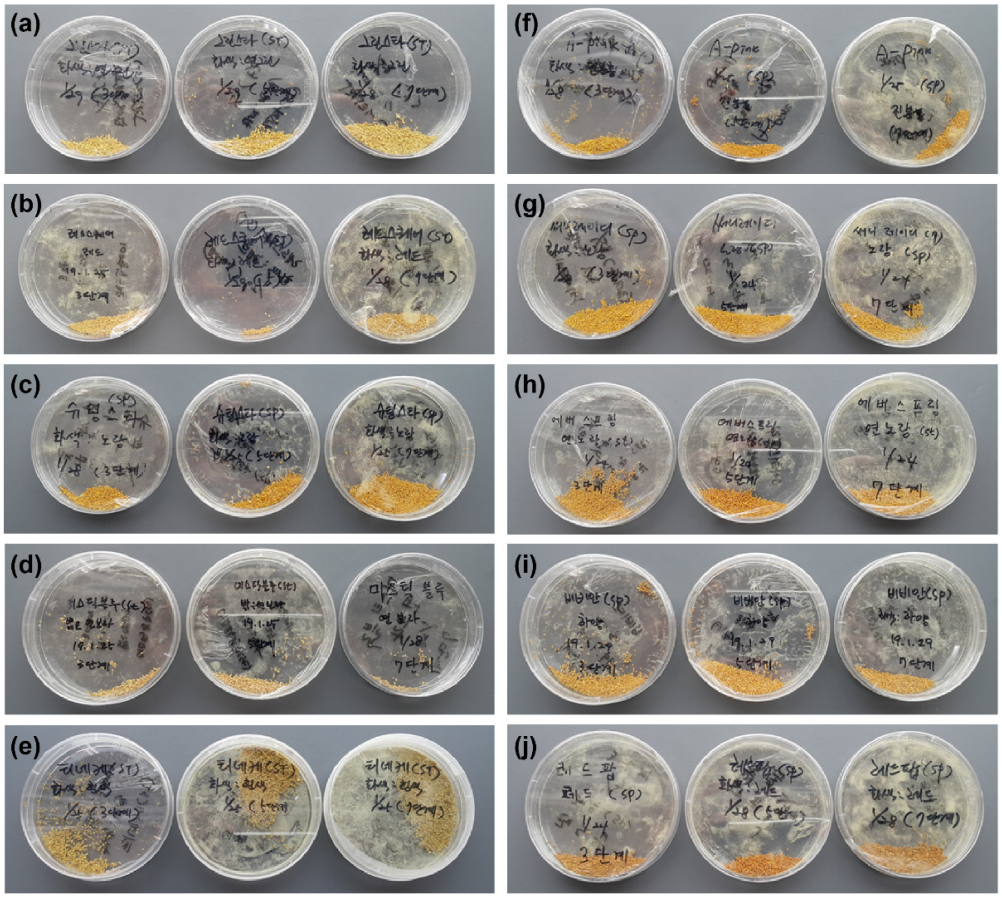

Figure 3. Comparison of pollen quantity from flowering stages 3, 5, and 7 (from the left). Standard type: (**a**) 'Green Star', (**b**) Red Square', (**c**) 'Shooting Star', (**d**) 'Mystic Blue', (**e**) 'Tineke'; spray type: (**f**) 'A-Pink', (**g**) 'Sunny Lady', (**h**) 'Ever Spring', (**i**) 'Vivien', (**j**) 'Red Pop'.

Pollen germination rates for each 3, 5, and 7 stages of flowering were 2.42–35.8%, 24.2–35.4%, and 24.9–34.8%, respectively, in the standard type, and 38.5–48.5%, 38.5–48.2%, and 37.8–49.5% in the spray type. Compared to the normal pollen rate, the pollen germination rate was relatively low, and the standard-type pollen germination rate was lower than that of the spray type. In the standard type, 'Mystic Blue' had the highest pollen germination rate and the other varieties were similar. In the spray type, 'Red Pop' had the lowest, and 'Vivien', 'A-Pink', and 'Sunny Lady' were similar. In the standard type, the pollen germination rate of 'Green Star' increased as the flowering progressed, but the pollen germination rate of 'Mystic Blue' collected at the beginning of the flowering was high (Figure 4). Further, in the spray type, 'Red Pop' had a higher germination rate from pollen in the early stage of flowering. There were no significant differences according to the flowering stage for each variety.

3.2. Characteristics of Pollen Vitality during Dry Period

Prior to anther storage, the dry conditions for vitro-pollination were analyzed. In the standard type, the normal pollen rate was 48.7–56.2% for 5 h of drying, 47.4–59.4% for 1 day, 47.3–59.2% for 3 days, and 49.4–58.5% for 5 days. In the spray type, the normal pollen rates following a drying period of 5 h, 1 day, 3 days, and 5 days were 64.2–76.8%, 60.8–80.6%, 57.8–78.4%, and 63.2–83.9%, respectively (Table 2). The longer the drying period, the lower the pollen germination rate was. According to the analysis of the difference in varieties, in the standard type, 'Green Star' had the lowest normal pollen rate in drying conditions on the third, fifth, and seventh days, and 'Shooting Star' had the highest normal pollen rate in all drying conditions. In the spray type, 'Ever Spring' had the lowest rate under conditions

of five hours, one day, and seven days of drying, and 'Sunny Lady' showed the highest pollen rate under all drying conditions. For both standard and spray types, there were no significant differences between the varieties relating to the drying time. There were no statistically significant differences relating to the drying time for all standard-type varieties; however, in 'Green Star', 'Mystic Blue', and 'Tineke', the normal pollen rate was high after seven days of drying. 'Red Pop' and 'Shooting Star' were high after one day of drying. Spray types had high normal pollen rates, with 'A-Pink' and 'Ever Spring' drying for five hours, 'Vivien' drying for one day, and 'Sunny Lady' and 'Red Pop' for seven days. In the 'A-Pink' and 'Sunny Lady' varieties, there was a statistically significant difference according to the drying time, and there was a difference in drying time depending on the variety.

Table 1. Percentages of normal pollen and pollen germination at stages 3, 5, and 7 of flowering.

Standard Type	Flowering Stages	The Percentage of				Spray Type	Flowering Stages	The Percentage of			
		Normal Pollen		Pollen Germination				Normal Pollen		Pollen Germination	
'Green Star'	3	46.4 ± 2.6 [z]	a [y]	24.2 ± 1.1	a	'A-Pink'	3	70.3 ± 1.5	a	48.5 ± 4.9	a
	5	47.2 ± 1.9	a	24.4 ± 3.5	a		5	72.6 ± 2.6	a	48.2 ± 2.5	a
	7	47.4 ± 2.8	a	25.8 ± 2.9	a		7	72.3 ± 3.0	a	49.5 ± 3.6	a
F value (p [x])		0.198 NS		0.220 NS		F value (p)		0.608 NS		0.046 NS	
'Red Square'	3	55.8 ± 2.6	a	26.5 ± 1.6	a	'Sunny Lady'	3	84.2 ± 3.1	a	50.4 ± 1.6	a
	5	57.4 ± 3.1	a	26.4 ± 1.9	a		5	80.6 ± 4.9	a	46.6 ± 3.1	a
	7	56.2 ± 0.1	a	24.9 ± 2.4	a		7	83.3 ± 3.7	a	46.8 ± 1.8	a
F value (p)		0.264 NS		0.735 NS		F value (p)		0.670 NS		2.459 NS	
'Shooting Star'	3	58.9 ± 3.0	a	25.4 ± 2.9	a	'Ever Spring'	3	63.1 ± 3.6	a	40.6 ± 2.6	a
	5	58.1 ± 1.8	a	24.2 ± 3.0	a		5	62.4 ± 4.1	a	40.1 ± 1.6	a
	7	58.5 ± 2.4	a	29.8 ± 4.1	a		7	65.9 ± 2.9	a	43.2 ± 4.0	a
F value (p)		0.100 NS		1.788 NS		F value (p)		0.411 NS		1.359 NS	
'Mystic Blue'	3	54.2 ± 3.5	a	35.8 ± 4.8	a	'Vivien'	3	74.8 ± 3.9	a	48.4 ± 1.9	a
	5	54.4 ± 4.4	a	35.4 ± 3.7	a		5	78.8 ± 3.3	a	46.2 ± 1.7	a
	7	54.2 ± 1.0	a	34.8 ± 1.8	a		7	74.4 ± 3.7	a	45.7 ± 3.4	a
F value (p)		0.107 NS		0.420 NS		F value (p)		1.577 NS		1.991 NS	
'Tineke'	3	46.7 ± 3.0	a	28.4 ± 1.7	a	'Red Pop'	3	69.8 ± 5.8	a	38.5 ± 3.7	a
	5	47.5 ± 2.5	a	27.8 ± 1.5	a		5	64.7 ± 4.8	a	38.5 ± 2.4	a
	7	51.8 ± 3.5	a	29.7 ± 1.5	a		7	72.5 ± 2.2	a	37.8 ± 2.8	a
F value (p)		2.483 NS		1.114 NS		F value (p)		2.533 NS		0.051 NS	
Total	3	52.29 ± 5.87	a	27.97 ± 4.75	a	Total	3	72.50 ± 7.84	a	45.51 ± 5.76	a
	5	52.94 ± 5.45	a	27.59 ± 4.78	a		5	71.75 ± 8.40	a	43.95 ± 4.44	a
	7	53.52 ± 4.26	a	28.79 ± 4.31	a		7	73.47 ± 6.38	a	43.35 ± 4.81	a
F value (p)		0.206 NS		0.262 NS		F value (p)		0.194 NS		0.221 NS	

a Mean separation within columns by Duncan's multiple range test at $p = 0.05$. [z] Mean ± standard deviation. [y] Mean separation within columns by Duncan's multiple range test at $p = 0.05$. [x] NS: Non-significant, respectively leveled by ANOVA.

The pollen germination rates for the standard type were 20.5 to 34.8%, 14.1 to 28.1%, 6.9 to 14.7%, and 2.2 to 11.2% for storage periods of five hours, one day, three days, and seven days, respectively. For spray types, the pollen rates were 36.5 to 50.6% for five hours of drying, 32.1 to 48.4% for one day, 19.4 to 27% for three days, and 10.1 to 19.7% for a seven-day period. The pollen germination rate decreased after three days. Even without considering the drying period, the spray type pollen germination rate was higher than that of the standard type. Analyzing the difference in varieties, we documented that standard types such as 'Mystic Blue' had a high pollen germination rate during all drying periods, indicating relatively higher pollen vitality. In the spray type, 'Red Pop' had a relatively low pollen germination rate compared to other varieties. The pollen germination rates of 'A-Pink' were relatively high within one day of drying, and 'Vivien' and 'Ever Spring'

were relatively high over three days. In both types, varieties differed significantly in drying times, and the shorter the drying time, the higher the pollen germination rate was. All 10 varieties differed in pollen germination rates according to the drying period. In the standard type, pollen germination rates were high within one day for 'Green Star' and 'Shooting Star', and the remaining varieties were high after drying for five hours. In the spray type, the pollen germination rate was high after drying within one day for 'A-Pink' and five hours for other varieties.

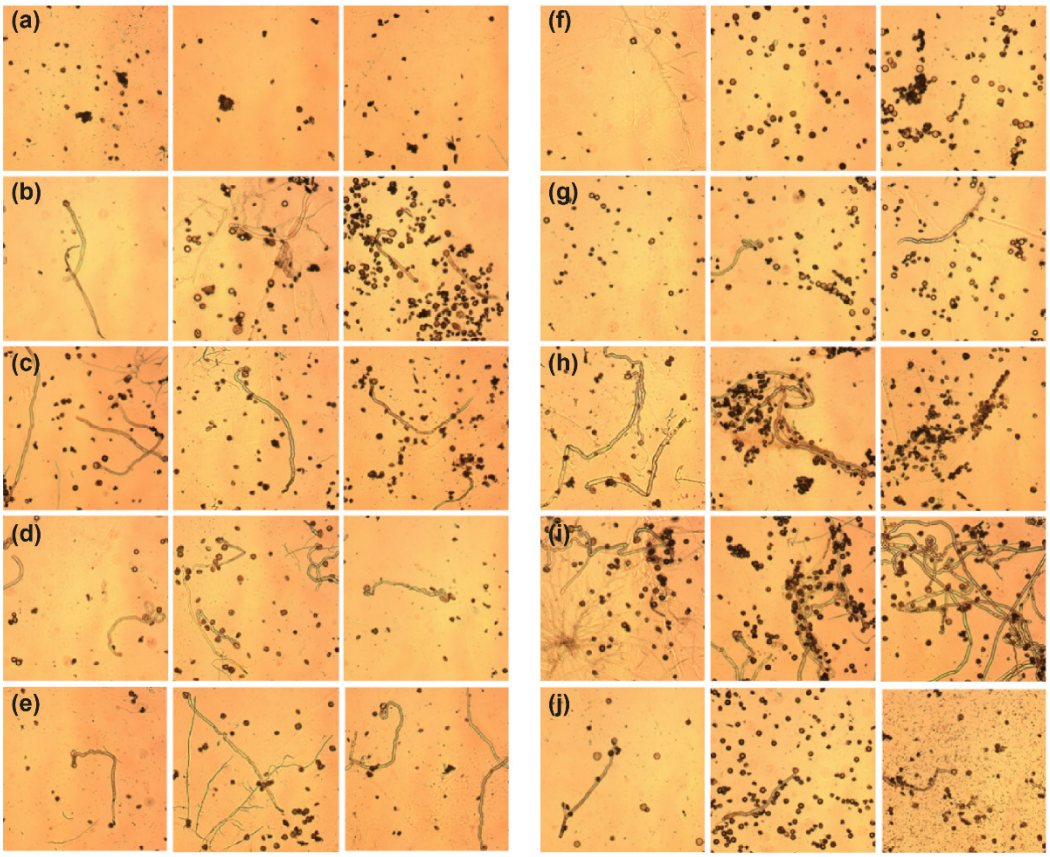

Figure 4. Comparison of pollen germination from flowering stages 3, 5, and 7 (from the left). Standard type: (**a**) 'Green Star', (**b**) Red Square', (**c**) 'Shooting Star', (**d**) 'Mystic Blue', (**e**) 'Tineke'; spray type: (**f**) 'A-Pink', (**g**) 'Sunny Lady', (**h**) 'Ever Spring', (**i**) 'Vivien', (**j**) 'Red Pop'.

3.3. Characteristics of Pollen Vitality at Storage Temperature

The normal pollen rate according to the pollen storage temperature in the standard type was found to be 43.8–55.6% at −72 °C, 42.6–56.7% at −20 °C, 44.7–55.8% at 0 °C, and 33.4–48.8% at 26 °C. This implies that, in the spray type, the rate was 53.8–67.5% at −72 °C, 53.3–69.6% at −20 °C, 50.6–69.6% at 0 °C, and 52.8–61.4% at 26 °C. The standard type had a relatively low percentage of pollen stored at 26 °C compared to pollen stored at temperatures below 0 °C. There were no differences in the normal pollen rate depending on the storage temperature of the spray type. The standard type differed significantly depending on the storage temperature, but there were no differences in the spray type. 'Tineke' and 'Green Star' were found to be sensitive to storage conditions owing to their relatively low normal pollen rates at all storage temperatures. 'Shooting Star' had a relatively high normal pollen rate. In the spray type, the normal pollen rate of 'Vivien' was relatively low at the storage temperature below 0°C, but it was relatively high at 26 °C. 'Red Pop' and 'Ever Spring' had relatively high normal pollen rates at all storage temperatures. When analyzed by variety, the normal pollen rate was higher when stored at a temperature below 0 °C than at 26 °C in all varieties. Significant differences were documented in all the varieties except 'Shooting Star', 'Ever Spring', and 'Vivien'. The standard cultivar 'Green

Star' had a normal pollen rate of 48.7% after five hours of pollen collection, but the normal pollen rate after 30 days of storage at 26 °C decreased by 15.3% to 33.4%. The normal pollen rate after 30 days of storage at −72 °C decreased by 4.9%. Additionally, the spray variety 'Sunny Lady' had a normal pollen rate of 76.8% after five hours of pollen collection. The normal pollen rate after 30 days storage at 26 °C decreased by 24.0% to 52.8%, whereas at −72 °C the rate decreased by 13.0% to 63.8%. To preserve normal pollen, storage at lower temperatures was found to be more advantageous than at room temperature.

Table 2. Effect of stage period of pollen on morphological condition and germination in five standard- and spray-type cultivars after 0.2, 1, 3, and 7 days storage.

Standard Type	Drying Time at Room Temp.	The Percentage of				Spray Type	Drying Time at Room Temp.	The Percentage of			
		Normal Pollen		Pollen Germination				Normal Pollen		Pollen Germination	
'Green Star'	0.2	48.7 ± 4.7 [z]	a [y]	22.6 ± 4.5	a	'A-Pink'	0.2	72.3 ± 2.6	a	50.6 ± 7.4	a
	1	48.2 ± 3.9	a	24.4 ± 1.2	a		1	70.5 ± 1.7	ab	48.4 ± 2.4	a
	3	47.3 ± 3.2	a	12.4 ± 1.3	b		3	57.8 ± 1.3	b	22.5 ± 0.7	b
	7	49.4 ± 2.1	a	4.2 ± 1.0	c		7	68.4 ± 4.7	c	10.1 ± 1.7	c
F value (p [x])		0.201 NS		42.419 ***		F value (p)		14.761 **		75.492 ***	
'Red Square'	0.2	54.8 ± 3.6	a	23.5 ± 1.5	a	'Sunny Lady'	0.2	76.8 ± 1.7	a	45.5 ± 1.5	a
	1	56.4 ± 3.7	a	19.4 ± 2.1	b		1	80.6 ± 2.8	a	41.1 ± 0.6	b
	3	55.7 ± 2.1	a	8.9 ± 1.8	c		3	78.4 ± 1.3	a	19.4 ± 2.2	c
	7	56.2 ± 4.5	a	2.2 ± 0.9	d		7	83.9 ± 1.5	b	14.2 ± 3.0	d
F value (p)		0.108 NS		100.946 ***		F value (p)		6.714 **		167.070 ***	
'Shooting Star'	0.2	56.2 ± 3.7	a	20.5 ± 2.4	a	'Ever Spring'	0.2	64.2 ± 3.9	a	44.4 ± 2.8	a
	1	59.4 ± 2.6	a	21.6 ± 3.9	a		1	60.8 ± 4.5	a	38.4 ± 1.5	b
	3	59.2 ± 6.5	a	14.2 ± 2.3	b		3	60.5 ± 1.7	a	27.1 ± 1.2	c
	7	58.5 ± 2.3	a	4.1 ± 1.1	c		7	63.2 ± 4.2	a	15.8 ± 1.3	d
F value (p)		0.361 NS		27.171 ***		F value (p)		0.646 NS		139.191 ***	
'Mystic Blue'	0.2	53.3 ± 1.9	a	34.8 ± 3.3	a	'Vivien'	0.2	76.5 ± 2.8	a	46.2 ± 1.6	a
	1	53.5 ± 3.0	a	28.1 ± 1.0	b		1	76.9 ± 4.8	a	35.4 ± 0.4	b
	3	54.2 ± 2.0	a	14.7 ± 1.8	c		3	69.4 ± 2.0	b	24.7 ± 2.4	c
	7	55.4 ± 5.1	a	11.2 ± 2.8	c		7	71.4 ± 1.7	ab	19.7 ± 2.2	d
F value (p)		0.294 NS		61.785 ***		F value (p)		3.332 NS		124.311 ***	
'Tineke'	0.2	49.8 ± 4.5	a	24.2 ± 2.1	a	'Red Pop'	0.2	70.5 ± 1.7	a	36.5 ± 1.0	a
	1	47.4 ± 4.5	a	14.1 ± 1.9	b		1	65.5 ± 2.9	a	32.1 ± 1.0	b
	3	50.2 ± 4.0	a	6.9 ± 1.5	c		3	68.4 ± 3.2	a	19.5 ± 2.6	c
	7	51.3 ± 4.4	a	3.1 ± 0.5	d		7	70.8 ± 4.4	a	14.1 ± 0.5	d
F value (p)		0.599 NS		96.129 ***		F value (p)		1.680 NS		138.869 ***	
Total	0.2	52.12 ± 4.76	a	25.11 ± 5.70	a	Total	0.2	71.78 ± 5.24	a	44.67 ± 5.73	a
	1	52.91 ± 5.71	a	21.52 ± 5.21	b		1	70.19 ± 7.32	a	39.36 ± 6.11	b
	3	53.27 ± 5.44	a	11.43 ± 3.50	c		3	66.87 ± 7.78	a	22.63 ± 3.46	c
	7	53.12 ± 4.76	a	4.99 ± 3.53	d		7	69.92 ± 7.17	a	14.86 ± 3.58	d
F value (p)		0.399 NS		60.535 ***		F value (p)		1.344 NS		123.357 ***	

a, b, c, d Mean separation within columns by Duncan's multiple range test at $p = 0.05$. [z] Mean ± standard deviation. [y] Mean separation within columns by Duncan's multiple range test at $p = 0.05$. [x] NS, **, ***: Non-significant or significant at $p \leq 0.01$ or $p \leq 0.001$, respectively leveled by ANOVA.

Analysis of the pollen germination rate showed that the standard type was 21.2–30.2% at the storage temperature of −72 °C, 20.4–28.7% at −20 °C, 11.1–19.4% at 0 °C, and 2.1–10.4% at 26 °C. In the spray type, 33.4–46.4%, 31.1–44.8%, 11.4–28.9%, and 3.7–17.4% were recorded at the temperatures of −72, −20, 0, and 26 °C, respectively. The lower the storage temperature, the higher the pollen germination rate was. When the storage temperature was below 0 °C, the spray type pollen germination rate was relatively higher than that of the standard type, but there were no differences between the types at 26 °C. When analyzed by variety, the pollen germination rate of 'Mystic Blue' was higher than

that of other varieties when the storage temperature was below 0 °C but was lower than that of other varieties at 26 °C. 'Mystic Blue' was more sensitive to storage temperature than other varieties. 'A-Pink' had a relatively higher pollen germination rate than other varieties at all storage temperatures. 'Red Pop' had a relatively low pollen germination rate at all storage temperatures. Significant differences were observed in both standard and spray types, depending on the variety and on storage temperature. The germination rate of pollen stored below −20 °C was high. When analyzing the storage temperature by variety, the pollen germination rate was high when stored at −72 °C and −20 °C in all varieties, and there were significant differences for all varieties. The standard variety 'Mystic Blue' had a 34.8% pollen germination rate five hours after pollen collection, but this plunged from 32.7% to 2.1% after 30 days of storage at 26 °C, and it was only 4.6% after 30 days of storage at −72 °C. In addition, the spray variety 'Red Pop' had a pollen germination rate of 36.5% after five hours of pollen collection. When stored at 26 °C for 30 days, the germination rate decreased by 32.8%, whereas, when stored at −72 °C for the same period, the germination rate decreased by only 3.1% (Table 3).

Table 3. Effect of storage temperature on normal pollen rate and germination rate for five standard and spray cultivars at 26, 0, −20, and −72 °C after 30 days of storage.

Standard Type	Treatment Temp.	The Percentage of				Spray Type	Treatment Temp.	The Percentage of			
		Normal Pollen		Pollen Germination				Normal Pollen		Pollen Germination	
'Green Star'	26	33.4 ± 1.2 [z]	b [y]	2.8 ± 0.6	c	'A-Pink'	26	52.8 ± 2.4	b	17.4 ± 2.0	c
	0	44.7 ± 3.2	a	19.4 ± 1.3	b		0	53.6 ± 1.9	b	25.9 ± 3.0	b
	−20	42.6 ± 2.3	a	22.1 ± 1.2	a		−20	57.9 ± 5.2	b	44.8 ± 2.4	a
	−72	43.8 ± 4.1	a	24.4 ± 2.2	a		−72	63.8 ± 1.6	a	46.4 ± 3.3	a
F value (p [x])		9.512 **		130.381 ***		F value (p)		7.458 *		79.115 ***	
'Red Square'	26	39.5 ± 1.5	b	7.4 ± 0.1	c	'Sunny Lady'	26	56.3 ± 2.4	c	10.8 ± 0.9	c
	0	48.2 ± 3.1	a	15.1 ± 3.0	b		0	68.5 ± 3.2	a	28.9 ± 2.2	b
	−20	46.9 ± 4.1	ab	22.1 ± 1.4	a		−20	65.0 ± 1.0	ab	38.7 ± 3.0	a
	−72	51.3 ± 5.3	a	22.7 ± 3.9	a		−72	63.1 ± 1.8	b	40.5 ± 2.3	a
F value (p)		5.150 *		22.331 ***		F value (p)		13.907 **		104.512 ***	
'Shooting Star'	26	48.8 ± 3.0	b	10.4 ± 2.4	b	'Ever Spring'	26	61.4 ± 4.5	b	14.7 ± 1.1	c
	0	55.8 ± 2.8	a	11.1 ± 3.2	b		0	69.6 ± 3.5	a	27.4 ± 1.5	b
	−20	56.7 ± 3.2	a	20.4 ± 1.9	a		−20	64.0 ± 4.4	ab	42.8 ± 2.1	a
	−72	55.6 ± 3.9	a	21.2 ± 2.2	a		−72	67.4 ± 0.9	ab	43.9 ± 2.3	a
F value (p)		3.662 NS		14.512 ***		F value (p)		2.673 NS		165.905 ***	
'Mystic Blue'	26	40.8 ± 1.6	b	2.1 ± 0.5	c	'Vivien'	26	58.4 ± 5.4	a	7.4 ± 0.1	c
	0	54.3 ± 3.3	a	15.8 ± 0.6	b		0	50.6 ± 2.9	b	24.7 ± 1.2	b
	−20	50.2 ± 1.6	a	28.7 ± 2.7	a		−20	53.3 ± 3.3	ab	40.9 ± 3.2	a
	−72	49.4 ± 5.4	a	30.2 ± 3.2	a		−72	53.8 ± 2.8	ab	39.8 ± 4.0	a
F value (p)		8.701 **		107.770 ***		F value (p)		2.718 NS		98.201 ***	
'Tineke'	26	33.4 ± 1.6	b	6.6 ± 1.0	b	'Red Pop'	26	57.9 ± 5.3	a	3.7 ± 0.5	c
	0	44.7 ± 2.3	a	11.4 ± 0.3	b		0	65.6 ± 4.1	a	11.4 ± 0.1	b
	−20	42.6 ± 0.4	a	21.9 ± 0.5	a		−20	69.6 ± 1.6	a	31.1 ± 2.8	a
	−72	45.8 ± 2.1	a	22.9 ± 3.4	a		−72	67.5 ± 3.5	b	33.4 ± 4.9	a
F value (p)		9.333 **		19.491 ***		F value (p)		5.572 *		81.384 ***	
Total	26	38.88 ± 5.92	b	5.89 ± 3.47	c	Total	26	57.35 ± 4.61	a	10.61 ± 5.24	c
	0	49.21 ± 5.37	a	14.59 ± 3.53	b		0	61.33 ± 8.69	a	23.49 ± 6.61	b
	−20	47.53 ± 5.70	a	23.15 ± 3.19	a		−20	51.88 ± 6.47	a	39.45 ± 5.30	a
	−72	48.91 ± 5.75	a	24.11 ± 4.57	a		−72	62.79 ± 5.64	a	40.45 ± 5.38	a
F value (p)		11.080 ***		78.202 ***		F value (p)		2.029 NS		94.855 ***	

a, b, c Mean separation within columns by Duncan's multiple range test at $p = 0.05$. [z] Mean ± standard deviation. [y] Mean separation within columns by Duncan's multiple range test at $p = 0.05$. [x] NS, *, **, ***: Non-significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively leveled by ANOVA.

As a result of analyzing the relationship between the normal pollen rate and the pollen germination rate, the Pearson correlation coefficient was 0.497 in the standard type and 0.304 in the spray type, indicating a significant difference in each type. There was a positive correlation between the normal pollen rate and the pollen germination rate. When analyzing the relationship between pollen germination rate and storage temperature, Pearson correlation coefficients were statistically significant at −0.788 and −0.866, respectively, depending on the type. The pollen germination rate had a negative correlation with the storage temperature (Table 4).

Table 4. Relationship between the percentage of normal pollen and the pollen germination rate after 30 days of storage at 26, 0, −20, and −72 °C.

Correlation Test	Type	r [z]	*p* [y]
Percentage of normal pollen × Percentage of pollen germination	Standard	0.497	0.000 ***
	Spray	0.204	0.118 NS
Storage temperature × Percentage of pollen germination	Standard	−0.788	0.002 ***
	Spray	−0.866	0.000 ***

[z] Pearson correlation coefficient. [y] NS, ***: Non-significant or significant at $p \leq 0.001$, respectively leveled by ANOVA.

3.4. Number of Pollinations and Fruiting Characteristics

Pollen of the 'Mishell' and 'Tineke' varieties used in copies was observed with a scanning electron microscope to investigate the effect of the number of pollinations on the fruit rate during rose hybridization. In both rose varieties, spherical normal pollen and wrinkled abnormal pollen were observed (Figure 5). The average diameter of pollen was 23.58 µm and 26.74 µm for 'Mishell' and 'Tineke', respectively. The number of anthers of 'Mishell' was 75.4, the normal pollen rate was 18.7%, the abnormal pollen rate was 81.3%, and the pollen germination rate was 24.8%. Also, the number of 'Tineke' anthers was 98.7, the normal pollen rate was 28.6%, the abnormal pollen rate was 71.4%, and the pollen germination rate was 32.7% (Table 5). Based on the FCR test, we found out that the ratio of normal pollen in both varieties was as low as 18%, and the pollen vitality was excellent in terms of the green fluorescence expression level of normal pollen.

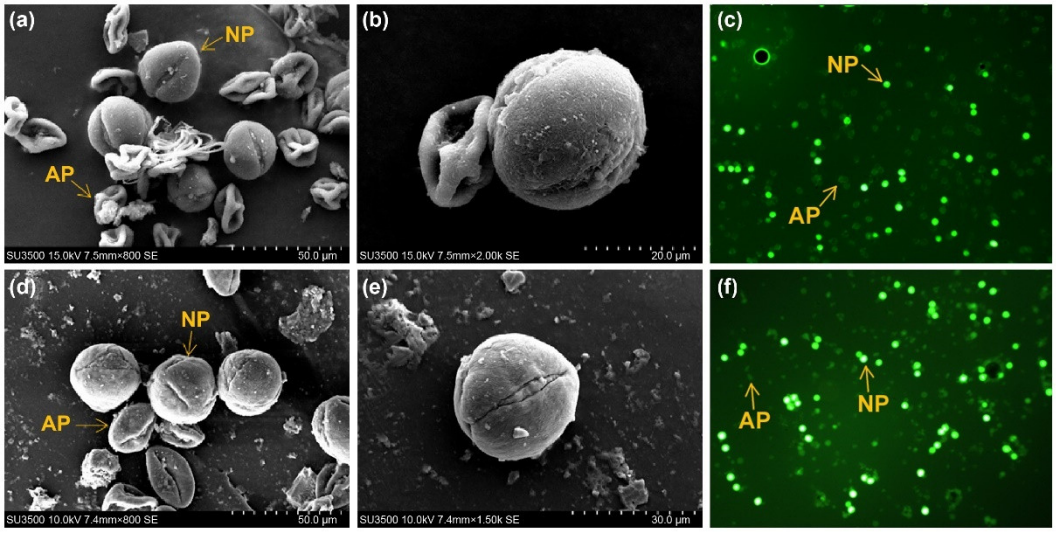

Figure 5. Scanning electron microphotographs of pollen shapes of 'Mishell' (**a**,**b**) and 'Tineke' (**d**,**e**). NP: normal pollen, AP: abnormal pollen. Light microphotographs of FCR test results for pollen germination of 'Mishell' (**c**) and 'Tineke' (**f**).

Table 5. Anther and pollen paternity characteristics.

Cultivar	No. of Anthers	Pollen Diameter [z] (μm)	Percentage of		
			Normal Pollen	Abnormal Pollen	Pollen Germination
'Mishell'	75.4	23.58 ± 4.19	18.7	81.3	24.8
'Tineke'	98.7	26.74 ± 2.76	28.6	71.4	32.7

[z] Three points observed under a microscope.

Fruit set percentage was investigated every 30 days after breeding by varying the number of pollinations (Figure 6). Falling occurred over time and the fruit set percentage continued to decrease after breeding. In the standard-variety 'Akito' × 'Mishell' combination, the fruit set percentage decreased sharply to 66.7% after 30 days and 33.3% after 60 days after a single pollination, whereas, in three pollinations, the fruit set percentage decreased to 88.9% after 30 days and 77.8% after 60 days. In the fifth pollination count, the fruit set percentage was found to be 77.8% after both 30 and 60 days and showed fewer or no falls. The spray-type 'Pink Charm' × 'Tineke' combination also showed a decrease of 66.7% after 30 days, 55.6% after 60 days, and 44.4% after 90 days of single pollination. The fruit set duration was maintained or decreased after 30, 60, and 90 days after three and five rounds of pollination. Our results are consistent with those reported by Zlesak [34], where a deficit in pollen yield from varieties with low fruit rates could be overcome by increasing the number of pollinations. In the spray-type 'Charming' × 'Tineke' combination, after 30 days, the fruit set percentage was 100% for five times, and 80% for one, two, and three times, but decreased to 55% for one and two times after 60 and 90 days and remained at 80% for three and five times.

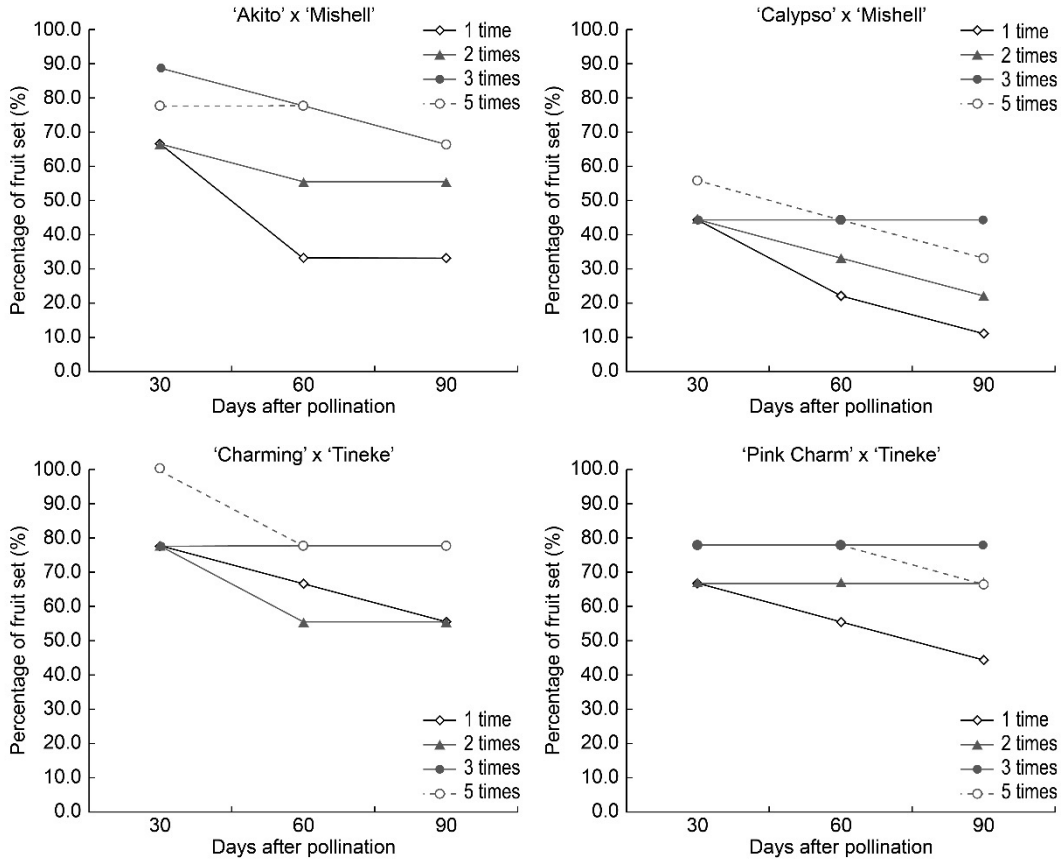

Figure 6. Effect of repeated pollination on fruit set percentage after 30, 60, and 90 days in two combinations of standard and spray types.

4. Discussion

4.1. Storage Properties Affecting Pollen Vitality

Pollen viability after anther dehiscence is extremely important for successful cross-breeding [24]. Storage conditions for pollen germination differ depending on the variety [35]. Therefore, this study analyzed the normal pollen rate and pollen germination rate in terms of the pollen collection stage, pollen drying time, and storage temperature to investigate the storage conditions for each variety in pollen management for rose breeding.

The collection of seeds for breeding was carried out in the third stage of flowering with 1–3 petals spread. There were no differences in the normal pollen rate or pollen germination rate based on the flowering stage. However, to achieve a larger pollen yield, we recommend harvesting at the seventh stage of flowering. Richer et al. [36] reported similar results, indicating that the germination rate of pollen collected by petals when the buds were three-quarters open was high.

The life expectancy of pollen in plant species depends on the dry resistance of pollen [37]. Pollen grains lose viability in both high and low humidity ranges, possibly due to rapid loss of water in the grain at low humidity and the physiological activity of the grain at high humidity [38]. Khosh-Khui et al. [22] explained that 50% relative humidity was appropriate to maintain the viability of rose pollen. Studies on various cultivars have indicated that pollen can be stored for a long time at low temperature and low relative humidity. [38–41]. In our study, there were no significant differences in the normal pollen rates in all varieties. The pollen germination rate showed a significant difference at the level of $p \leq 0.001$ in terms of the drying period, and the pollen germination rate was the best at five hours of drying. When the drying period was longer than three days, the pollen germination rate decreased to less than half compared to the standard of five hours. Drying is an important factor in pollen preservation as it can prevent microbial growth and extend shelf life by minimizing moisture-mediated degradation reactions [42]. However, as the drying period increases, the relative humidity decreases, causing problems in pollen vitality. Therefore, drying is essential to maintain the viability of the pollen without reducing the germination power of pollen.

The viable level of pollen in fresh roses varied depending on the variety and was related to the conservation temperature [10]. Based on our analysis of changes in pollen vitality with changes in storage temperature, and despite the different tendencies of each variety, we documented that the normal pollen rate was generally higher at temperatures below 0 °C than at room temperature. When rose pollen was stored at room temperature, the germination power decreased rapidly [43]. However, the in vitro germination effect of cryopreserved pollen was found to be similar to that of fresh pollen [44]. In our study, the pollen germination rate was high at sub-zero storage temperatures in all varieties of standard and spray types ($p \leq 0.001$), but there were no significant differences between −20 °C and −72 °C in all varieties. The vitality of rose pollen lasted for a long time under drying and low-temperature conditions. The pollen vitality decreased at hyperhumidities and high temperatures, and pollen germination decreased significantly after four to five days at temperatures above 25 °C [25]. Macovei et al. [24] also explained that pollen in roses can be efficiently stored at −20 °C for up to three months and Marchant et al. [11] demonstrated that rose pollen stored at low temperatures for eight weeks leads to no reduction in fertility levels. Rajasekharan and Ganeshan [45] explained the possibility of low-temperature storage of rose pollen by reporting that low-temperature storage of rose pollen can maintain viability and fertility status for one year. Additionally, we documented a positive correlation between the normal pollen rate and the pollen germination rate in both standard and spray varieties (Table 4). When pollen was collected and stored in advance, storing it at low temperatures was effective in maintaining pollen vitality. In previous studies, cryogenic storage was useful for maintaining the vitality of pollen, but studies have shown that pollen can be stored not only in a cryogenic freezer but also in a freezer that can maintain −20 °C, so it can be economically advantageous to maintain it up to −20 °C.

4.2. Characteristics of Fruiting by Pollination

The pollen viability of male parents is an important factor influencing the success of cross-pollination [46]. The male parents 'Mishell' and 'Tineke' were in the form of normal pollen with a spheroidal shape. A spheroidal shape, unlike a branching shape, can resist physical irregularities caused by random collisions in the pollination process. The spheroidal shape results in reduced ultraviolet damage and water desorption owing to the smaller surface area, which is advantageous for pollen grains [47]. The shape and structure of pollen correlate with the growth habitat and pollen biology [48,49].

The higher the number of pollinations, the lower the variation in the fruit set percentage over time. However, the fruit set percentage did not differ due to the number of pollinations (three and five) and was found to be high at three pollinations. If the fruit set percentage is high, more pollen can be obtained and used for breeding. Therefore, increasing the number of pollinations during breeding resulted in an increase in the percentage of fruit set, which is advantageous in increasing the breeding efficiency. Considering the economic aspects of the cost and time of breeding, three breeding times can be expected to be appropriate.

5. Conclusions

This study was conducted to develop a method for managing rose pollen that can increase breeding efficiency. For the management method, pollen vitality was analyzed through the treatment of the anther collection, considering the flowering step, pollen drying time, and pollen storage temperature. The effect of the storage method was explained by analyzing the fruit rate through a crossbreeding experiment with pollen according to the specified storage conditions. The normal pollen rate and pollen germination rate of the pollen obtained in flowering stages 3, 5, and 7 were not significantly different. However, since the amount of pollen was the highest at the seventh stage, it is advantageous to collect anthers at the seventh stage of flowering to obtain a lot of pollen. The optimal pollen drying time for pollen germination was five hours after the anther was opened, and as the drying time increased, the pollen vitality significantly decreased. When dried pollen was stored at low temperatures of −20 °C and −72 °C for 30 days, the germination rate of the pollen was similar to or slightly lower than immediately after pollen collection. However, the germination rate of pollen stored at an ordinary temperature (26 °C) was very low. It is effective to store pollen below 0 °C. The pollen collected at the third stage of flowering was dried for 5 h and then stored at −20 °C for 30 days before hybridization. The percentage of normal pollen in the two varieties of male parents used in breeding was about 18%, but the vitality of normal pollen was high. As the number of pollinations increased, the fruit set percentage tended to increase, and there was no significant difference between the third and fifth times. Considering the economic aspects of time and cost of breeding, the appropriate number of pollinations to increase the number of seeds is three.

This study suggested a method for efficiently collecting and utilizing pollen from a specific type of genetic source. The results of this study may be helpful in breeding roses that reflect consumption trends by collecting pollen from various individuals to increase the fruiting rate of crossbreeding. To increase the fruiting rate after pollination, it is important not only to manage the pollen of the male parents but also the form of the hypanthium used as the female parents. A follow-up study is required on the form of hypanthium and the crossability of the female parents that produce good results.

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