Estimating Successful Cross Combinations Using Fertility Indexes in Roses

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Abstract

The success of rose breeding programs is low due to poor seed sets and germination rates. Determining fertile parents and cross combinations that show high compatibility could increase the effectiveness of breeding programs. In this study, three rose varieties belonging to *Rosa x hybrida* (Jumilia, First Red and Magnum), and two old garden rose species (Black Rose and Cabbage Rose) with known ploidy levels were reciprocally cross-bred under controlled conditions to determine the successful crosses by checking fertility. The pollen germination rate (PG), crossability rate (CR), seed number per fruit (SNpF), seed production efficiency (SPE), seed germination rate (SGR), fruit weight (FW), seed weight (SW), stigma number (SiN), etc. were recorded. Compherensive fertility index value was calculated. PCA, a correlation matrix and hierarchical heat map were used to evaluate the data. The findings showed that old garden roses had more viable pollen than hybrid tea roses. The crossing success improved as pollen fertility increased. Also, female parent fertility improved crossing success just as much as pollen fertility. Although the pollen fertility and stigma numbers were low, some combinations had a higher CR and SPE. The maximum SPE was determined in combinations where Black Rose (BR) was the female parent (8.67% to 19.46%) despite the lower stigma number and low pollen fertility. The highest CR was recorded in the BR x First Red (FR) (94.36%). All combinations in which BR was used as the female parent had a more stable CR. The SNpF of combinations where hybrid rose varieties were female parents and old garden roses were pollen parents was higher than other combinations where hybrid rose varieties were both female and pollen parents. The SPE in intraspecific crosses was lower than that obtained from interspecific crosses. Moreover, the SGR decreased in combinations that produced heavier seeds. The results suggested that SPE is a more accurate parameter than SNpF in demonstrating combination success in breeding programs. BRxFR, BRxJ, BRxM and BRxCR combinations can be used successfully, as the PCA analysis and heat map showed. BR showed better performance as both seed and pollen parents according to the comprehensive fertility index. From the correlation matrix, it is understood that the number of stigmas will not be an important criterion in parent selection. Old garden roses can be used as parents to increase the success of breeding programs. However, it is necessary to reveal how successful they are in transferring the desired characteristics such as scent, petal number and color.

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Introduction

The rose (*Rosa* spp.) is one of the most popular plant species grown worldwide (*Kılıç, 2020*). It is the most commercial ornamental plant, and the production of cut roses is number one among all rose-productive areas. Millions of cut roses of different colors, shapes, and types are produced in more than 50 countries every year (*Trademap, 2022*). Moreover, consumer demands are constantly changing, and thousands of new varieties are developed by breeding companies each year (*Doğan, 2022*). It is imperative for companies to develop new rose varieties to maintain their market share (*Uran, 2022*).

Taxonomic diversity in roses offers breeders the opportunity to be successful in the development of new varieties (*de Vries & Dubois, 1988*). The way to benefit from this diversity in the most efficient way is crossbreeding, so this is the most commonly used breeding method for developing new rose varieties (*Liorzou et al., 2016*). However, roses are known for their difficult sexual reproduction, from pollination to seed set and germination (*Perez & Moore, 1985; Gudin, 1992; Abdolmohammadi et al., 2014; Doğan et al., 2020*). The meiotic abnormalities and accumulation of deleterious recessive alleles due to the interspecific origin and the intensive inbreeding performed in the past has reduced success in cross breeding and represents the economic risk of breeding programs. Moreover, self- and cross-incompatibility, which are widely prevalent in varieties of rose with low pollen quality and seed dormancy, reduce the efficiency of breeding programs (*Debener, Janakiram & Mattiesch, 2000; Pipino et al., 2013a*). According to rose breeding studies, some cross combinations do not produce fruit or seeds, and even when they do, the seed germination rate is very low, with some seeds not germinating at all (*Farooq et al. 2016; Khan et al. 2020*). However, there are some combinations in which fruit set and seed germination rate can reach 100.0% (*Grossi & Jay, 2002*; *Atram et al., 2015*), the seed number can reach 100 (*Turna, 2022*). A similar trend was found for pollen fertility. It has been stated that pollen germination rates show a wide variation from 0% to 99.0% depending on genotype and climactic conditions (*Pipino et al.*, *2011*; *Giovannini et al., 2017*).

Considering that the development of new varieties in rose breeding programs takes from 4 to 10 years and the chance of developing new varieties is from 0.002% to 0.003% (*Chaanin, 2003*), rose breeders aspire to increase the efficiency of a breeding programme by producing more offspring. Therefore, successful parent selection is of great importance. They must know the probability of producing large viable numbers of seeds in the species and varieties, as well as the compatibility of combinations, the desired characteristics, the ability to transfer the desired characteristics according to the breeding goals like scent, petal number and flower stem length to the next generation, pollen viability and germination rates. Knowing pollen parent fertility and the compatibility of cross combinations is needed to improve the breeding programme efficiency and the chance of developing new varieties by increasing the seed set rate and seed germination rate. Scientific studies on the success of crossing old garden roses and their ability to transfer characteristics to the next generation are limited. The lack of sufficient information is a great challenge for both researchers who will carry out breeding studies and amateurs who will just start breeding roses.

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108   There are thousands of rose varieties and hundreds of rose species. With such a large number
109   of rose genotypes to choose from, defining their fertility becomes difficult, and selecting
110   potential varieties with characteristics takes years (*Khan et al., 2021*). Moreover, there is very
111   little scientific research conducted on the success of crosses in roses because it is mainly carried
112   out by commercial companies, and key information is kept as a trade secret. Modern roses have
113   the desired characteristics in terms of marketability, but their more complex genetic backgrounds
114   suggest that their fertility is lower than old garden roses. The crosses between old garden roses
115   and modern roses are thought to create more successful combinations than crosses among
116   modern roses. In addition, cross incompatibility may be more common in modern roses. This
117   study was designed to determine parental fertility, the incompatibility of roses, and cross
118   combinations that show relatively good performance to obtain a good seed set and viable seed
119   rate by checking fertility and compatibility. Enhancing the success rate of hybridization among
120   the existing gene pool of *Rosa* species is essential.

121

Materials & Methods
123   Crossbreeding on roses were conducted during 2020 and 2021 in a polyethylene (Roof cover
124   system-180 micron) and polycarbonate (Forehead and side coating-8 mm) plastic-covered
125   greenhouse belonging to the Department of Horticulture in the Agriculture Faculty of Ankara
126   University based in the province of Ankara, Turkey [(39°57'53.8"N 32°51'50.8"E (*Google Maps,*
127   *2020*)]. Studies on pollen viability and germination rates of rose species/varieties were conducted
128   in the cytology laboratory of the Department of Horticulture at Ankara University at the same
129   time as the crossbreeding.

130

Plant Material
132   The plant material consisted of three of the most widely known global varieties of hybrid tea
133   roses (Jumilia, First Red and Magnum) which belonging to the *Rosa x hybrida,* and two different
134   old garden rose species known as Black Rose (*Rosa odorata* Louis XIV, BR), and Cabbage Rose
135   (*Rosa centifolia* L., CR). Hybrid tea roses are the most popular group among the modern roses
136   widely used today. They are firm favorite in the cut flower sector with their repeating blooms,
137   standard type, large and semi-double or double flowers (usually 25 to 30, but up to 80 in some
138   hybrids) and long flower stems. Old garden roses are not generally preferred as ornamental
139   plants except for gardens because they do not meet commercial quality criteria. They are of
140   industrial importance due to their intense scent (*Kılıç, 2020*). While modern roses have the
141   desired higher visual quality, old garden roses have a higher pollen quality than modern roses.
142   Some floral characteristics recorded in the study, such as the petal and stigma numbers of the
143   roses, are given in Table 1.
144   It was important that the ploidy levels were the same in the selection of the parents. All
145   species and varieties were tetraploid with 2n=4x=28 chromosome numbers, and the DNA
146   content varied between 2.33 pg/2C and 2.54 pg/2C [*Kılıç, 2020; Kazaz et al., 2022, unpublished*
147   *data*) (Table 1).

Commented [DMFk5]: Objective need to be defined and must be revised

148   All of the rose genotypes used as parents were purchased as 1-year-old seedlings from the
149 Şanlıurfa-based company Atılım in 2017 and brought to the greenhouse, which had a double-row
150 bed system 40 cm high and 20 cm wide. After planting the seedlings in horizontal bags (100 x 20
151 x 12 cm, 24 liters) containing the cocopeat, they were placed on beds with a width of 80 cm.
152 Each horizontal bag contained six plants. During the vegetation period, the greenhouse
153 temperature was kept at 23-30ºC, and the relative humidity was kept at 60%-70%. To prevent the
154 plants from being damaged by the high light intensity, a heat-shade curtain providing 55% shade
155 was used. The water and nutrients were given to the plants by a drip irrigation system, and the
156 system was automatically controlled by a fertigation computer. During the vegetation period, the
157 number of irrigations was adjusted based on 30% drainage rate. The amount of water per drip
158 was generally adjusted to be from 80 cc to 100 cc. The nutrient solution given by *Mercurio*
159 *(2007)* was used for fertilizing the plants and the electrical conductivity (EC) of the solution
160 given to the plants was kept to be from 1.5 to 1.8 mS/cm in the early stages of development, 1.8
161 to 2.0 mS/cm in the following periods, and the pH was from between 5.3 to 5.8 (*Hazar and*
162 *Baktır, 2013*). The formulation of the nutrient solutions is given in Table 2. Pesticides and
163 biological control agents were used against diseases and pests. Pesticides were applied to the
164 plants until 7 days before and 15 days after the cross. In the biological control, predator mite
165 (*Phytoseiulus persimilis*) was used against red spiders.
166
167 **Crossbreeding**
168 Reciprocal hybridization (cross pollination) was performed between varieties and species from
169 May 15 to June 30. A total of 20 combinations were formed, and 30 crosses were produced for
170 each combination. Firstly, emasculation was performed on the flowers selected as the female
171 parents of all varieties when one-third of the flowers were opened at 08.00 am. Then they were
172 covered with a paper bag (*Crespel & Mouchotte, 2003; Chimonidou et al., 2007*). Anthers taken
173 during emasculation were used as the pollen parents, and pollen grains were expected to be
174 released from the anther after one day in a growth chamber at a temperature of 24 ºC and 60 %
175 humidity. Twenty-four hours after the emasculation of flowers and collection of anthers, the
176 pollen was dispersed and was rubbed onto the stigma with a brush (*Jacob & Ferrero, 2003;*
177 *Spethmann & Feurerhahn, 2003*) and again covered with a paper bag for four days. Labels with
178 combination codes were attached (*de Vries & Dubois, 1983; Gudin, 2003*). Hybridizations were
179 formed both in the apical bud and in the flowers formed on the shoots of the axillary buds.
180   After the last date of hybridization (140 days), fruits that reached harvest maturity defined as
181 when the color of the fruit changes from green to an orange-red color, and browning begins on
182 the flower stalk, were harvested. The number of fruits was counted, and the crossability rate (%,
183 CR) per combination (fruit set rate) was determined. The average fresh weight per fruit (FW) for
184 each combination was also recorded using an Ohaus NV 212 model precision weighing machine.
185 Then, the fruits were brought under laboratory conditions, the fruits slit with a sharp knife and
186 the seeds were separated from the fruit to determine the average number of seeds per fruit for
187 each combination (SNpF). The average fresh weight per seed was also recorded (SW) for each

188 combination. Moreover, the data on the number of flowers crossed, fruit and seed sets that
189 making up the fertility indexes were used to calculate the average crossability rate for a parent
190 (ACR), the percentage high crossability for a parent (PHC) and seed production efficiency
191 (SPE).
192   The ACR for a parent was calculated as the sum of cross compatibility rates in specific
193 crosses divided by the number of cross-combinations involving that particular parent (*Mondo et*
194 *al., 2022*):
195

$$ACR\ (\%) = \frac{\sum Crossability\ rates}{Number\ of\ cross\ combinations} x\ 100$$

196
197
198   The PHC for a parent was calculated as the number of times the cross compatibility rate
199 exceeded the species' overall cross compatibility divided by the number of cross combinations in
200 which that parental genotype was involved (*Mondo et al., 2022*):
201

$$PHC\ (\%) = \frac{Number\ of\ crossability\ rates > overall\ mean}{Number\ of\ cross\ combinations} x\ 100$$

202
203
204   The SPE for a cross was calculated as the number of viable seeds divided by the number of
205 stigmas of the female parent in that cross combination (the expected number of seeds in rose
206 fruits is equal to the stigma number) and the number of pollinated flowers multiplied by 100
207 (*Mondo et al., 2022*):
208

$$SPE\ (\%) = \frac{Number\ of\ viable\ seed\ set}{Number\ of\ flowers\ pollinated\ x\ stigma\ number} x\ 100$$

209
210
211   The selected F_1 hybrid seeds from the fruit were treated with moist cold stratification at
212 $4\pm1°C$ for 100 days immediately after seed weights were recorded (*Gudin et al., 1990; Debener*
213 *& Mattiesch, 1996*). Perlite was used as the stratification medium, and seeds were treated using a
214 fungicide with 25% tebuconazole as the active ingredient against fungal diseases for thirty
215 minutes. The seeds were then placed in zip-top bags containing perlite, and placed in cold
216 storage. After the moist cold stratification, seeds were sown in vials containing peat and
217 germinated in a plastic-covered greenhouse (at a temperature from 18 to 21°C) and were
218 irrigated by the fogging irrigation method during the germination process. When the seeds
219 showed cotyledon and hypocotyl growth above the growing medium, they were considered to
220 have germinated (*Nadeem et al., 2015*; *Khan et al., 2020*). The germinated seeds were counted,
221 and the seed germination rate (%, SGR) was determined using the following formula:
222

$$SGR\ (\%) = \frac{Number\ of\ seeds\ germinated}{Number\ of\ seed\ sown} x\ 100$$

223
224

Evaluation of pollen quality

The pollen viability and germination rate of all rose species/varieties were determined. Anthers taken from flowers of all rose species and varieties during emasculation were placed in glass bottles and brought to the laboratory. The bottles were kept overnight in a growth chamber in the laboratory for anther dehiscence (in darkness at a temperature of 24 ºC and 60 % humidity). The pollen's viability and germination rate were determined using dispersed pollen from anthers as soon as the pollens were removed from the chamber. The IKI (iodine potassium iodide) method was used to determine the pollen viability rates, and the petri dish method was used to determine the germination rates (PG). The IKI was applied according to *Doğan et al., (2020)*. Five minutes after being treated with the IKI solution, pollen grains were counted under the microscope. Pollen grains dyed black and dark brown were considered viable, pollen grains dyed orange, red or light brown were considered semi-viable, and pollen grains dyed yellow or colorless were considered non-viable. Fifty percent of the pollen grains categorized as semi-viable were accepted as viable. The petri dish method was modified according to *Kazaz et al., (2020)*. A 2mm layer of germination medium containing 20% sucrose, 10 ppm boric acid and 1% agar solution was poured into plastic petri dishes. Before freezing, the solution in petri dishes was divided into 4 separate areas, and pollen was sprinkled lightly on each area with the help of a brush. The preparation, which was incubated for 8 hours at a temperature of 24ºC and 60% humidity, was removed taken from petri dishes and then germinated pollen grains were counted under the microscope. Pollen grains were considered germinated when they formed a pollen tube longer than their own diameter. In both methods, the Leica DM1000 model microscope and imaging system with x40 and x100 magnification objectives lenses were used for the pollen count.

Data analysis

The experiments on crossbreeding and pollen quality were established in a completely random design with three replications (Wasonga *et al.,* 2020). In the crossbreeding, ten crosses were made in each replication. In the pollen viability test, two coverslips were used for each rose variety, and counts were done in four areas on each coverslip. In the pollen germination test, counting was performed in four areas over two slices chosen randomly in each petri dish. In both methods, averages of 250 pollen grains were counted per area. Statistical analysis was performed using IBM SPSS Statistics version 20.0 software. An analysis of variance was applied to the angularly transformed data.

The mean differences were established using Duncan's test (where $p \leq 0.05$) (Kılıç *et al.*, 2020). The original data on fertility indexes of F1 hybrids was recorded and processed using Microsoft Office Excel 2021 and XLSTAT. To provide a comprehensive evaluation of fertility, the method specified by *Wang et al. (2022)* was used. The fertility indexes for genotypes used as parents and cross combinations were normalized through variable transformation (min-max scaling), and their weight coefficients were calculated using AHP (the consistency rates of parents and combinations were 0.0052 and 0.0004, respectively). Moreover, a heat map was created to

265 visualize hierarchical clustering and standardized the values for each combination. Principal
266 component analysis was also performed, and a biplot was established for greater approximation
267 than the coefficient of correlation using XLSTAT in order to determine relationships among the
268 fertility indexes (*Evgenidis et al., 2011*).
269

Results

Pollen viability and germination rate

272 The analysis of variance showed that there was a statistically significant difference between
273 pollen parents for pollen germination and pollen viability rates (p≤0.05). First Red (51.97%)
274 showed the highest pollen viability rate, whereas the lowest viable pollen rate was recorded for
275 Jumilia (39.56%). The difference between Black Rose and Magnum, Cabbage Rose, and First
276 Red was found to be statistically insignificant. Similar to viable pollen rates, the highest pollen
277 germination rate was found in Black Rose (44.22%), followed by Cabbage Rose (32.70%),
278 whereas the lowest germination rate was recorded for Jumilia (15.65%) (Figure 1). First Red had
279 31.37% more viable pollen than Jumilia. The germination rate of Black Rose pollen grains was
280 2.83 times higher than Jumilia.
281

Fertility indexes

283 The results of the statistical analysis showed that the crossability rate, the average number of
284 seeds per fruit for each combination, the average fruit and seed weight per combination, the seed
285 germination rate, the seed production efficiency, the average crossability rate, and the percentage
286 of high crossability varied significantly among all cross-combinations (Table 3) and
287 species/varieties (Table 4) (p≤0.05).
288 In the study, 600 crosses were created, and a fruit set was achieved in 51.16% of them. The
289 highest crossability rate was observed in BR x FR (94.36%), which was in the same statistical
290 group as BR x J (90.75%) and CR x J (80.0%). However, the crossability rate was above 65.0%
291 in all combinations where BR was the female parent. The lowest crossability rate was recorded
292 in J x FR (8.70%), and there was no statistical difference between it and J x M (11.68%), M x FR
293 (12.52%), and CR x M (23.12%). Moreover, the crossability rate changed from 32.0% to 60.0%
294 in combinations where BR was the pollen parent, 46.10% and 68.81% where CR was the pollen
295 parent, 48.54% and 90.75% where J was the pollen parent, 11.68% and 76.95% where M was the
296 pollen parent and 8.70% and 94.36% where FR was the pollen parent (Table 3). The crossability
297 rate of BR x FR was 10.9 times higher than J x FR combinations.
298 The maximum seed number per fruit was determined to be in FR x CR (19.31 pcs), and it was
299 not statistically different from M x BR (16.51 pcs) and M x CR (17.45 pcs). The lowest SNpFs
300 were found in J x M (2.12 pcs), J x FR (3.46 pcs), BR x M (4.53 pcs), CR x BR (5.31 pcs) and
301 BR x CR (5.84 pcs) combinations, respectively, and they were not significantly different. The
302 SNpF varied between 5.31 and 16.51 in combinations where BR was the pollen parent, 5.84 and
303 19.31 where CR was the pollen parent, 6.42 and 12.40 where J was the pollen parent, 2.12 and

304 9.60 where M was the pollen parent and 3.41 and 9.80 where FR was the pollen parent (Table 3).
305 The seed number per fruit of FR x CR was 9.2 times higher than J x M combinations.
306   The heaviest fruit was observed in J x BR (14.60 g), and the lightest fruit was recorded in CR
307 x J (2.67 g). These crosses were in the same statistical group as all combinations where FR and
308 BR were female parents. Average fruit weight ranged from 3.20 g to 14.60 g in combinations
309 where BR was the pollen parent, 3.56 g to 10.29 g where CR was the pollen parent, 2.67 g to
310 6.38 g where J was the pollen parent, 2.77 g to 3.81 g where M was the pollen parent, and 3.06 g
311 to 4.35 g where FR was the pollen parent (Table 3). The combination with the highest seed
312 weight was M x CR (88.06 mg). It was in the same statistical group as CR x BR (74.67 mg), J x
313 BR (64.20 mg), CR x FR (61.46 mg), BR x CR (57.44 mg), and in all combinations where FR
314 was the female parent. The lowest seed weight was determined in BR x FR (31.66 mg). Average
315 seed weight changed from 47.40 mg to 74.67 mg in combinations where BR was the pollen
316 parent, 38.33 mg to 88.06 mg where CR was the pollen parent, 39.41 mg to 73.24 mg where J
317 was the pollen parent, 38.98 mg to 75.38 mg where M was the pollen parent and 31.66 mg to
318 61.46 mg where FR was the pollen parent (Table 3). While the M x CR combination provided
319 64.05% heavier seeds than the BR x FR combination, 81.72% lighter fruit was obtained from the
320 CR x J combination compared to the J x BR combination.
321   The maximum seed germination rates were observed in J x M (47.73%) and BR x FR
322 (41.73%). The minimum seed germination rate was recorded in J x BR (8.78%), which was not
323 statistically different from CR x J (9.14%), CR x BR (12.79%), FR x M (13.42%), and CR x FR
324 (14.21%). The seed germination rates varied from 8.78% to 36.84% in combinations where BR
325 was the pollen parent, 30.69% to 36.40% where CR was the pollen parent, 9.14% to 39.08%
326 where J was the pollen parent, 13.42% to 47.73% where M was the pollen parent and 14.21% to
327 41.73% where FR was the pollen parent (Table 3). Seeds from J x M combinations germinated at
328 a rate 5.44 times higher than seeds from J x BR combinations.
329   The highest seed production efficiency was found in BR x FR (19.46%), followed by BR x J
330 (17.20%), whereas the lowest seed production efficiency was seen in J x M (0.18%), which was
331 in the same statistical group as J x FR (0.22%) and M x FR (0.51%) and all combinations where
332 FR was the female parent. The seed production efficiency ranged from 1.05% to 4.03% in
333 combinations where BR was the pollen parent, 2.23% to 10.01% where CR was the pollen
334 parent, 1.34% to 17.20% where J was the pollen parent, 0.18% to 8.67% where M was the pollen
335 parent and 0.22% to 19.46% where FR was the pollen parent (Table 3). The seed production
336 efficiency of BR x FR combinations was 108.11 times higher than J x M combinations.
337   In combinations in which BR was the female parent, 1.45 to 2.49 times more fruit set was
338 achieved, 1.09 to 2.85 times more germinated seeds were obtained, and greater seed production
339 efficiency was found, 4.16 to 9.96 times compared to the combinations in which other genotypes
340 were female parents. In general, fruit set rate, seed set rate and seed production efficiency were
341 found to be approximately 2x, 1.5x, 1.5x, and 5x higher, respectively, in combinations where
342 old garden roses were used as parents than in cross combinations among modern roses. In
343 combinations where old garden roses were used as pollen parents, the seed set rate was found to

344 be 1.5x higher than in combinations in which modern roses were used as pollen parents, while
345 the crossability rate and seed production efficiency decreased by 1.2x. In combinations where
346 old garden roses were used as female parents, the crossability rate and seed production efficiency
347 were found to be 2x and 3.6x higher, respectively, than in combinations in which modern roses
348 were used as female parents.
349   Among the parents, the maximum ACR rate of 64.53% and the maximum SPE rate of 41.51%
350 were obtained from the Black Rose. The Magnum had the lowest ACR rate of 38.67%, while
351 First Red had the lowest SPE rate of 4.17%. Jumilia and First Red showed a higher PHC rate
352 than the other three parents. (Table 4).
353
354 **Principal component analysis, correlation matrix, and hierarchical clustering heat map**
355 A principal component analysis (PCA) was carried out and a biplot was established for a better
356 approximation than the coefficient of correlation to describe the crossability success between
357 and/or within rose species and varieties. Given an eigenvalue larger than 1, the first (F1), second
358 (F2), and third (F3) principal components accounted for 38.28%, 20.88%, and 16.28% of the
359 total variation, totaling 75.44%. The PCA-biplot results indicated a positive correlation between
360 pollen germination, fruit weight, seed weight and seed number per fruit because they were placed
361 on the same side and had similar vector lengths. Higher crossability rates, seed production
362 efficiency, and seed germination rates were found in all cross-combinations where the Black
363 Rose was the female parent compared to other combinations. The stigma number and seed
364 weight were significant parameters in all combinations, where First Red was the seed plant with
365 high values of these traits (Figure 2).
366   The correlation matrix supported the relationships obtained by PCA. When the correlation
367 matrix is examined in Table 5, it is seen that there is a positive correlation between crossability
368 rate and seed production efficiency, between seed weight and stigma number, between fruit
369 weight and seed number per fruit, between fruit weight and pollen germination rate, and between
370 seed number per fruit and stigma number. Additionally, it was determined that there was a
371 negative relationship between seed weight and seed germination rate and between stigma number
372 and seed production efficiency.
373   The heat map analysis based on the fertility indexes of different cross combinations divided
374 the examined traits into two main groups. While stigma number, pollen germination rate, seed
375 and fruit weight, and number of seeds per fruit were included in the 1st major group, fruit set rate,
376 seed production efficiency, and seed germination rate parameters were included in the 2nd major
377 group. Cross combinations were also basically divided into two main groups hierarchically. The
378 1st major group was located on the left side of the map where predominantly positive tendencies
379 were represented by green color tones and negative tendencies were represented by red color
380 tones cross combinations in which Black Rose was used as a female parent. They were grouped
381 in a single cluster showing higher values for seed production efficiency, seed germination rate
382 and crossability rate compared to all other cross combinations. The 2nd major group was divided
383 into two subgroups. It was seen that the combinations in the 1st subgroup consisted of

384 combinations in which hybrid tea roses were used as female and old garden rose as pollen
385 parents. Despite the low stigma number, Black Rose provided higher seed production efficiency.
386 Similarly, the group with the higher stigma number had lower seed production efficiency (Figure
387 3)..
388

389 **Comprehensive evaluation of the fertility of the cross combinations and parents**
390 The composite fertility index of parents and cross combinations are shown in Figure 4. The
391 comprehensive fertility index values of combinations varied from 0.14 to 0.89, making it easier
392 to identify combinations with greater fertility. While the highest comprehensive fertility index
393 was found in ' Black rose x First Red' and 'Black Rose x Jumilia', the lowest fertility index was
394 determined in the 'Jumilia x First Red'. The comprehensive index values of female parents varied
395 from 0.26 to 0.82. While the highest comprehensive fertility index was found in Black Rose as
396 the female parent, the lowest fertility index was determined in Jumilia and Cabbage rose.
397 Magnum and First Red had better comprehensive fertility indexes than Jumilia. The
398 comprehensive index values of parents as pollen parent varied from 0.13 to 0.90. While the
399 highest comprehensive fertility index was found in Cabbage rose as the pollen parent, the lowest
400 fertility index was determined in Magnum. After the Cabbage rose, Black rose became the
401 second pollen parent with the highest fertility index. Magnum and First Red had better
402 comprehensive fertility indexes than Jumilia.
403

404 **Discussion**
405 **Pollen viability and germination rate**
406 Pollen productivity and pollen quality of pollen parents are included in crossbreeding because
407 they are essential in terms of fertilization success and hybridization efficiency. Pollen viability
408 and germination rate parameters, which express pollen quality, must be high in rose species and
409 varieties for successful fertilization. Various studies have been carried out by many researchers
410 to determine the pollen quality of rose species and varieties. *Pipino et al.* (*2011*) reported that the
411 germination rates of 11 different hybrid tea roses were between 0% and 46.5%. *Nadeem et al.*
412 (*2013*) showed that the viability rates of 13 different hybrid tea roses were between 35.0% and
413 70.0%, and the germination rates were between 1.3% and 46.5%. *Erbaş, Alagöz & Baydar,*
414 (*2015*) stated that in *R. damascena* Mill., pollen viability rates in different flowering periods
415 were between 32.8% and 71.5%, and germination rates were between 24.2% and 57.0%.
416 According to *Żuraw et al.* (*2015*), the pollen viability rates of four different rose species ranged
417 from 26.7% to 96.9%. *Giovannini et al. (2017)* reported that the germination rates of 44 different
418 hybrid tea roses varied between 6.0% and 99.0%. Khan et al. (2021) stated that the viable pollen
419 rates of some Rosa x hybrida varieties were between 28.60% and 67.40%, and the germination
420 rates were between 6.90% and 67.40%.
421   In this study, the pollen viability rate varied between 39.56% and 51.97%, while the pollen
422 germination rate varied between 15.65% and 44.22%. Although the findings of this study are
423 generally similar to the results obtained in the studies mentioned, the lower and upper limit

424   values changed. It is thought that pollen quality varies depending on the genotype, ploidy levels
425   (*Uckert, 2014*), the methods used (*Sulusoglu & Cavusoglu, 2014*), the climatic conditions, the
426   nutritional status of the plant, the time of the pollen is collected (season, flowering period, and
427   development period of flowers) (*Martins et al., 2017*), and the storage conditions and storage
428   duration (Miler *& Wonzy, 2021*). Moreover, similar to the current study results, it has been stated
429   that wild and old garden roses have higher pollen quality than hybrid roses (Ueda *& Hirata,
430   1989; Gudin & Arene, 1991; Zlesak, Zuzek & Hokanson, 2007; Kazaz et al., 2020*). The low
431   pollen fertility of hybrid roses could be related to interspecific hybridizations, meiotic
432   abnormalities, heterozygous polyploidy parents, and the accumulation of lethal recessive alleles
433   (*Nadeem et al., 2013*). One of the reasons why old garden roses are so fertile is their ability to
434   produce more morphologically normal pollen. The fact that their germination ability is better
435   than that of all modern roses may be related to the fact that old garden roses are more resistant to
436   dehydration than modern roses. It has been reported that the ability to form morphologically
437   normal pollen in roses and the resistance to dehydration of pollen may vary depending on the
438   species and varieties (*Pacini & Dolferus, 2019*). Another reason is that their capacity to produce
439   2n pollen may be higher than that of modern roses. *Gao et al., (2019*) indicated that 2n pollen is
440   involved in hybridization and has a competitive advantage while it traverses the stigma and
441   enters the style, and 2n pollen production varies according to genotype in roses.
442   The pollen quality of the same species/varieties differed because of the chemical and
443   biological test methods, and the viability rates obtained by the IKI method were found to be
444   higher than the germination rates obtained by the agar method in petri dishes. In a study by
445   *Parfitt & Ganeshan (1989)*, it was determined that chemical methods did not show similarities
446   with biological methods. Generally, it is expected that there will be a linear relationship between
447   pollen viability and germination rate (*Martins et al., 2017*). However, higher viability rates can
448   be seen compared to the results obtained by biological methods since pollen that has not yet
449   matured can be dyed using chemical methods (*Şensoy et al., 2003*). On the other hand, pollen
450   may retain the capacity to metabolize while losing the ability to germinate (*Gaudet et al., 2020*).
451   In this study, it was also determined that First Red and Magnum were better than Jumilia in
452   terms of pollen germination rates. This may be because First Red and Magnum are much older
453   than Jumilia. It may be related to the pollen quality of modern roses in varieties developed
454   recently being lower than in older varieties due to the inability to overcome the narrow gene pool
455   problem. Some researchers have stated that hybrid roses are incompatible due to inbreeding.
456   Breeders in the past have used a much smaller number of fertile varieties that produce above-
457   average offspring (*de Vries & Dubois, 1988*).
458
459   **Fertility indexes**
460   One of the most important factors affecting success in rose breeding is the fertility of the female
461   parent. While high viability and pollen germination rates are desired in the pollen parents, high
462   fertilization rates, fruit set, seed formation, seed production efficiency, and seed germination
463   rates are expected in the female parents. However, there was variation among the combinations

464 in terms of fruit set, seed formation, seed production efficiency, seed and fruit weight, and seed
465 germination rate.
466   In this current study, the crossability rate varied from 8.70% to 94.36% among the
467 combinations, and the average crossability rate was 51.16%. The number of seeds per fruit
468 changed from 2.12 to 19.31. In previous research, it was reported that the rate of fruit set in
469 modern roses is generally less than 50% (*Gudin, 2003*), and because of hybridization between
470 old garden and modern roses, the rate of fruit set decreased to 25% (*Gudin, 2000*). However,
471 *Khan et al.* (*2021*) determined that the fruit set rate was 63.33% in their crossbreeding study,
472 regardless of the combination. *Abdolmohammadi et al. (2014)* stated that the fruit set rate varied
473 from 0% to 80.00% among the combinations. In the study by *Atram et al. (2015)*, it varied
474 between 0% and 100.0%, and it changed over a range from 30.00% to 83.00% in the *Nadeem et*
475 *al.* (*2015*) studies. In studies where the average number of seeds per fruit was determined, it was
476 stated that roses have an average of 0 to 50.0 seeds per fruit (*Zlesak, 2007*). However, *Pipino et*
477 *al. (2011)* reported that the average number of seeds per fruit in hybrid tea roses varied from 1.10
478 to 21.30. *Abdolmohammadi et al. (2014)* discovered that the average number of seeds per fruit
479 varied from 0 to 35.30 due to crosses between modern roses and wild and old garden roses.
480 *Nadeem et al. (2015)* determined that the average number of seeds per fruit ranged from 15.0 to
481 33.0 in combinations using modern roses as parents. *Farooq et al. (2016)* reported that the
482 average number of seeds per fruit varied from 0 to 17.0 in crosses between 5 different rose
483 species. *Khan et al. (2020)* stated that the seed number per fruit ranged from 0.0 to 15.0 among
484 hybrid roses.
485   The difference in crossability rate among combinations may be related to the complex genetic
486 structures of genotypes (*Ueckert, 2014*), parental fertility (*Nadeem et al., 2015*), incompatibility
487 (*MacPhail & Kevan, 2009*), meiotic abnormalities and accumulation of lethal alleles (*Ogilvie et*
488 *al., 1991*; *Nadeem et al., 2015*). Moreover, hormonal control affecting the embryo and hip
489 formation may cause a low crossability rate (Cruden *& Lyon 1989; Stone, Thompson & Dent-*
490 *Acosta, 1995*). *Gudin (2000)* reported that the development of embryos seems to control the fruit
491 development in roses. In this current study, a positive relationship was found between seed
492 production efficiency and crossability rate. The low crossability rate could be attributed to cross
493 incompatibility since most tetraploid roses are self-compatible (*Rajapakse et al., 2001*).
494 According to *Zlesak* (*2007*), hybrid roses are mostly self-pollinated, and the success of their
495 crossing is dependent on the ability of the female gametes to admit foreign pollen (*Nadeem*
496 (*2012*). Among the tetraploid parents used in this study, combinations with low fruit and seed set
497 despite a high pollen germination rate were found where there were combinations with a high
498 fruit and seed set using the same pollen parent. Another reason for the variations in the
499 crossability rate and seed set may be related to petal numbers. It has been reported that roses with
500 a low number of petals form more fruits than those with a higher number of petals, and the
501 number of fruits and seeds is high in the wild, and old garden roses have low petal numbers due
502 to the increase in fertility (*Baydar et al., 2016*). Researchers also reported that the number of
503 fruits and seeds decreased due to sterility depending on the decrease in the number of anthers as

504   the petal numbers increased. In this current study, the lowest number of petals was found in the
505   Black Rose, which showed the highest fruit set rate and had the highest seed production
506   efficiency, which agrees with the study by *Baydar et al. (2016)*. Other reasons are considered for
507   the lower crossability rate and seed set. *Love et al. (2016)* reported that the diameter of the
508   stigma affects the success of crossbreeding, and the number of seeds may vary according to the
509   amount of pollen on the stigma, although it has been determined that an increase in the amount
510   of pollen on the stigma improves the fruit set rate and the number of seeds (*Falque et al., 1995*)
511   while an excessive amount of pollen on the stigma can decrease the number of seeds (*Lankinen,*
512   *Lindström & D'Hertefeldt, 2018*). *Ogilvie et al. (1991)* argued that the pollen tube growth barrier
513   could cause decreased seed set.
514   Fruit weight and seed weight are important quality parameters in the evaluation of breeding
515   success, and they differed from each other between studies. In this study, fruit weight from
516   between 2.67 g to 14.60 g, whereas seed weight varied from 31.66 mg to 88.06 mg. However,
517   *Nadeem et al. (2013)* found that the maximum fruit weight was 4.89 g. *Ercişli & Eşitken (2004)*
518   indicated that the fruit weights of different rose species varied from 3.12 g to 5.20 g. *Farooq et*
519   *al. (2016)* discovered that fruit weights varied from 0 g to 2.0 g when five different rose species
520   were cross-bred. *Khan et al. (2021)* determined that fruit weight ranged from 0 g to 5.63 g
521   among hybrid combinations. The study by *de Vries et al. (2000)* showed that fruit weight varied
522   from 3.50 g to 14.30 g in their hybridizations with roses at different temperatures. Pipino et al.
523   *(2011)* stated that the average seed weight of roses was 66.30 mg. *Doğan (2022)* determined that
524   the seed weight of crosses between miniature roses and different rose species and varieties varied
525   from 40 mg to 166 mg. *Turna (2022)* found that the seed weight of roses changed over a range
526   from 120.0 mg to 260.0 mg. In some studies, it has been determined that there is a positive
527   relationship between fruit weight and the average number of seeds per fruit (*Khan et al., 2021;*
528   *de Vries & Dubois, 2000*). In this current study, no relationship was found between fruit weight
529   and seed number or seed weight. It is considered that fruit weight varies depending on the
530   genetic structure of the female parent, and the fruit weight may be largely related to the thickness
531   of the fruit flesh. Moreover, Pipino et al. (2013b) indicated that tetraploid hybrids have a similar
532   seed and hip development under the same climatic conditions. Contrary to what was stated by the
533   researchers, fruit and seed development differed under the same conditions in this current study.
534   This indicates that the genetic structure of the female parent is more effective than the climatic
535   conditions.
536   Generally, it has been reported that seed germination rates in roses vary from 30% to 45%
537   (*Leus et al., 2018*). In this study, the germination rate varied from 8.78% to 47.73%. However,
538   *Grossi & Jay* (*2002*) found that the seed germination rate ranged from 0% to 100% depending on
539   the ploidy levels of the parents, and the average seed germination rate was determined to be
540   14.21% in 112 different hybrid combinations using modern roses as the female parent and both
541   modern and wild roses as the pollen parent. *Pipino et al.* (*2011*) determined that the seed
542   germination rates of 11 different hybrid tea roses ranged from 15.4% to 37.1%. It was
543   determined by *Ueckert (2014)* that the seed germination rate ranged from 10.6% to 62% in

544   hybrid combinations created with genotypes with different ploidy levels obtained through
545   intraspecific and interspecific hybridization. *Abdolmohammadi et al. (2014)*, because of
546   hybridisation studies between old garden and modern roses, found that the seed germination rate
547   ranged from 0% to 93.40%, and the average seed germination rate was 43.41%, depending on
548   the ploidy levels of the parents. *Uran (2022)* stated that seed germination rates of miniature rose
549   x cut rose combinations ranged from 0.00% to 47.50%, but the average germination rate was
550   found to be 10.73%.

551   The reasons for the differences in the seed germination rate of combinations could be
552   dormancy (*Alp et al., 2009*), plant physiology and morphology [genotype number of female
553   organs, etc.; (*Australian Government, 2009*)], in addition to the complex genetic structures of
554   genotypes, parental fertility, incompatibility, meiotic abnormalities, and accumulation of lethal
555   alleles (*Ogilvie et al., 1991; Nadeem et al., 2015*). Genetically sterile hybrid seeds may have
556   formed when haploid or triploid pollen grain production occurs in tetraploid pollinating parents.
557   Alternatively, the fact that the response to many applications (hot and cold stratification
558   temperatures and times, etc.) for the elimination of dormancy varies depending on the species
559   and varieties (*Alp et al., 2009*), which may have caused differences in the seed germination rate
560   of the combinations. *Lammerts* (*1946*) reported that the breeding of roses is occasionally
561   hampered by premature abortion of the developing embryo, resulting in few or no viable seeds.
562   Because roses are highly heterozygous, this behavior reduces the efficiency of breeding
563   programs and genetic understanding (*Gudin & Mouchotte, 1996*).

564   As seen above, many hybridization studies have been conducted on roses, and it has been
565   stated that important parameters such as fruit set rate, the average number of seeds per fruit, and
566   seed germination rate vary considerably among the hybrid combinations (*Gudin, 2000; Nadeem*
567   *et al., 2013; Nadeem et al., 2015; Fibrianty & Kurniati, 2019*). There are similarities between
568   previous studies and this study, but there are differences between the lower and upper limits in
569   terms of parameters. The reasons for the differences in the lower and upper limits could be the
570   complex genetic structures of genotypes, ploidy levels *(Ueckert, 2014)*, parental fertility
571   (*Nadeem et al., 2015*), incompatibility, meiotic abnormalities, accumulation of lethal alleles,
572   climatic conditions (*MacPhail & Kevan, 2009*), dormancy (*Falque et al., 1995; Khan et al.,*
573   *2020*), stratification methods for seeds, plant physiology and morphology, and pollination
574   methods (*MacPhail & Kevan, 2009*).

575   In this current study, the average number of seeds per fruit of some combinations obtained
576   under *in vivo* conditions was in parallel with the pollen germination rate obtained under *in vitro*
577   conditions. However, the average number of seeds per fruit and the seed production efficiency of
578   some combinations were reduced despite using a pollen parent that had a high pollen
579   germination rate. *Pipino et al. (2011)* indicated that, in some cases, the *in vitro* germination
580   ability may not fully reflect the *in vivo* germination ability. It is thought that this may be related
581   to meiotic abnormalities or pre-pollination barriers in roses. Tetraploid pollen parents are
582   expected to produce pollen grains with a diploid (2n) genome. However, sometimes, due to the
583   abnormalities that occur during the meiotic division of the pollen mother cell, haploid (n) and

584 triploid (3n) pollen grains may also occur besides diploid pollen grains. Since the callus plates of
585 diploid pollen grains are thinner than those of haploid pollen grains, these pollens are more likely
586 to fertilize the egg by moving more easily and faster in the stigma. However, the pollen tube of
587 haploid pollen grains is shorter than the pollen tube of diploid pollen grains and may not reach
588 the ovary within the time required to fertilize the egg cell (*Gao et al., 2019*). Therefore, the rose
589 genotypes used as pollen parents in this current study may have produced haploid pollen grains,
590 and haploid pollen grains germinating *in vivo* may not have shown the same performance under
591 in vitro conditions. If incompatible, the pollen grains cannot germinate on the stigma, or even if
592 they germinate, they cannot develop in the stigma and reach the ovary (*Karaağaç & Kar, 2016*).
593 Therefore, every germinated pollen grain in roses should not be expected to produce seeds,
594 whether due to meiotic abnormalities, pre-pollination barriers or incompatibility.
595   Differences were observed between the parents in terms of ACR, PHC and SPE. The ACR of
596 old garden roses as parents were higher than that of hybrid tea roses. Old garden roses and
597 Magnum showed more stable crossability and were closer to average. The SPE was higher in the
598 Black Rose. These results indicate that the species or varieties with a high crossability rate as the
599 female parent may not always show high seed production efficiency. However, the crossability
600 rate and seed production efficiency of combinations had positive correlations with each other
601 according to PCA and the correlation matrix. These varying relationships between parents and
602 combinations underline the importance of pollen fertility. The ACR and PHC indices provided
603 an overall parental evaluation without distinguishing between female and pollen parents.
604 However, the comprehensive fertility index provided a more effective evaluation that
605 distinguishes between the female and pollen parents. The comprehensive fertility index of female
606 parents indicated that Black Rose was the best female parent followed by Magnum and First
607 Red, in accordance with the SPE value given in Table 4. Furthermore, the comprehensive
608 fertility index supported the PHC and ACR values. The PHC was mostly affected by the seed
609 parent according to the comprehensive fertility index and this means may be that parents with
610 high fertility are more stable crossability and were closer to average. The lower ACR in
611 Magnum, First Red and Jumilia had a lower comprehensive index value as a pollen parent,
612 which may indicate that ACR value is more affected by pollen parent.
613   The correlation matrix, PCA and hierarchical clustering heat map results indicated that seed
614 weight is negatively correlated with seed germination rate and positively correlated with the
615 stigma number. The negative relationship between seed weight and seed germination rate may
616 indicate that germination does not occur because of the decrease in water absorption and air
617 diffusion due to the increase in seed coat thickness and the prevention of embryonic expression.
618 *Mohapatra & Rout (2005)* attributed a low germination rate to mechanical restrictions such as a
619 hard pericarp, which prevents embryo expansion. According to *Phat et al. (2015)*, weak
620 embryos, thick seed coats, and larger air spaces cause poor seed germination. According to the
621 correlation matrix, there was also a negative correlation between the stigma number and SPE.
622 This is because the Black Rose, which has a lower stigma number compared to other species and
623 varieties, produces seeds at similar rates. Although First Red had ten times the number of

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stigmas as the Black Rose, the average number of seeds per fruit of First Red was only twice that of the Black Rose. This shows that there are some important differences between the number of seeds per fruit and the seed production efficiency.

As a result, old garden rose species had a higher pollen germination rate than commercial modern roses. Crossing success improved as pollen fertility increased. However, female parent fertility improved the crossing success as much as pollen fertility. Although the pollen fertility and stigma number were low, those combinations had higher crossability rates and seed production efficiency. The maximum seed production efficiency and crossability rate were determined in combinations where BR was the female parent, despite the lower number of stigmas and low pollen fertility. They also had a more stable crossability rate. The SNpF of combinations where hybrid rose varieties were female parents and old garden roses were pollen parents was higher than other combinations of the same female parent. This appeared to be related to both the stigma number and the greater incompatibility between the modern rose varieties. Moreover, the seed germination rate decreased in combinations that produced heavier seeds. It is predicted that it may be related to the seed coat and/or be caused by disruptions in the development of the endosperm and embryo. Fruit set rate, seed production efficiency and the average number of seeds per fruit in interspecific crossing were higher than those obtained in intraspecific crossing. Simultaneously, pollen quality in hybrid roses was lower than in old garden roses. This suggests a stronger pre-pollination barrier in intraspecific crosses than in interspecies crosses. The presence of a higher seed germination rate in intraspecific hybridization suggests a stronger post-pollination barrier in interspecific hybridization. The results indicated that the pre-pollination and post-pollination isolation mechanisms of old garden and hybrid roses differ from each other. Although Magnum had a higher pollen germination rate than Jumilia, showing a lower comprehensive fertility index as pollen parent made the incompatibility more visible in roses. The germination rate of seeds obtained from combinations of old garden roses and the germination rate of seeds obtained from combinations of hybrid tea roses did not differ from each other. However, the presence of post-pollination barriers in old garden roses will increase the chance of obtaining more hybrids using the embryo rescue method. In crosses between modern roses, this chance is low as there is more of a pre-pollination barrier. Although it is a fact that the chance of seed set increases with the higher number of stigmas in modern roses, the seed set rate did not decrease in parallel with the number of stigmas in old garden roses despite the lower number of stigmas and even gave close results. Seed production efficiency is related to cross-compatibility rather than the number of stigmas and pollen germination rates.

This is the first study to show the crossing success of the BR. Moreover, there was no study about seed production efficiency in rose breeding studies. It is thought that this parameter is important for the evaluation of breeding studies.

Conclusions

The results showed that cross combinations including old garden roses, can increase the success of breeding programs. Old garden roses, especially Black Rose, as seed and pollen parents, have

Commented [DMFk7]: Conclusion should be precise with success of findings

improved fruit and seed set rates. As female parents, Magnum and First Red appear to be relatively productive hybrid tea roses. The comperehensive fertility index, hierarchical clustering heat map and PCA showed that BR x FR, BR x J, BR x M, and BR x CR combinations can be used successfully. The PCA suggests that SPE is a more accurate parameter than SNpF in revealing combination success in breeding programs. Seed production efficiency should be demonstrated in future breeding studies. The results obtained from the Comprehensive fertility index may indicate that Magnum and First Red cultivars can create more successful combinations as maternal parents and cabbage rose as paternal parents. This study will contribute to breeding programs because cross combinations could be determined using the available data on parental performance. The combinations selected this way are more likely to be successful than a random choice. Moreover, parental selection and determination of the cross combinations expends a lot of cost, labor, and time in the crossbreeding programs. Increasing the parent gene pool makes a great contribution and is convenient for breeders. The high-volume hybridization strategy used by better-funded international breeding programs cannot be repeated by many public-sector national breeding programs. To improve the effectiveness of their breeding programmes and provide more new varieties to their market, they can quickly adopt the method of creating fewer, more careful selections of cross combinations and parents.

Acknowledgements

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