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The Influence of Inbreeding on *Petunia hybrida*

Leyla Cheheltanan, Ali Tehranifar*, Mahmoud Shoor, Hossein Nemati, Saeed Khosravi

Department of Horticultural Science and Landscape, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

*Corresponding author's email: tehranifar@um.ac.ir

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ABSTRACT

Inbreeding influences ornamental traits by increasing homozygosity, often leading to inbreeding depression, particularly in cases of self-fertilization. This study aimed to evaluate the effects of inbreeding on the expression of traits in five genotypes of petunia. Plants were cultivated from seeds of *Petunia hybrida* grandiflora burgundy, *Petunia hybrida* grandiflora blue, *Petunia hybrida* supercascade white, *Petunia hybrida* supercascade red, and *Petunia hybrida* milliflora rose morn, along with seeds of three successive selfing generations (S1, S2, and S3). The experiment was conducted in a greenhouse at Ferdowsi University of Mashhad (2021-2023) and aimed at comparing different generations across the five genotypes. The results revealed that inbreeding significantly reduced flower diameter, flower count, germination percentage, and total flavonoid and anthocyanin contents. These reductions became more pronounced with successive inbreeding generations, with the most substantial decreases observed in S3. Conversely, inbreeding led to increases in corolla tube length, stem height to the first flower, and mean germination time. Among the genotypes, *Petunia hybrida* supercascade white was the most affected by selfing due to inbreeding, with reductions of 26.17%, 71.32%, and 77.77% in flower diameter, flower count, and germination percentage, respectively.

Abbreviations: First generation resulting from self-pollination (S1), Hybrid (F1), *Petunia hybrida* grandiflora Burgundy (P1), *Petunia hybrida* grandiflora Blue (P2), *Petunia hybrida* supercascade White (P3), *Petunia hybrida* supercascade Red (P4), *Petunia hybrida* milliflora rose morn (P5), Second generation resulting from self-pollination (S2), Third self-pollinated generation (S3).

Introduction

Petunia hybrida is one of the most popular bedding plants globally, widely used for ornamental purposes (Firdous et al., 2022). This commercial plant is an artificial hybrid developed in the 19th century by crossing *P. axillaris*, a species with white, moth-pollinated flowers, and *P. interior*, characterized by pink, bee-pollinated flowers (Segatto et al., 2014). Beyond its ornamental value, petunia has become a significant model system for genetic and physiological studies due to its short life cycle, simple cultivation requirements, and ease of propagation (Firdous et al., 2022). Petunia is naturally self-incompatible, with gametophytic self-incompatibility controlled by a multi-allelic S locus. Similar to other plants in the Solanaceae family, petunia can overcome self-incompatibility before sufficient accumulation of S-RNase, particularly through pollination of immature

pistils. This technique allows the production of homozygous S alleles (Robbins et al., 2000).

Inbreeding arises from natural or artificial mating between related individuals or those with some level of genetic relationship (de Freitas et al., 2016). Selfing, the most extreme form of inbreeding, reduces heterozygosity by approximately 50% generation^{-1}. This reduction often leads to phenotypic changes associated with inbreeding depression (Carr and Dudash, 1997). There is a positive correlation between inbreeding depression and levels of selfing, with increased selfing typically resulting in more severe inbreeding depression (Liu et al., 2021). Inbreeding depression occurs when self-pollination increases the frequency of harmful homozygous alleles, reducing overall fitness and desirable traits in offspring. While inbreeding can sometimes fix beneficial alleles and improve genetic value, it also increases homozygosity for deleterious alleles, contributing to the loss of heterozygosity and inbreeding depression (Labroo et al., 2023).

The genetic basis of inbreeding depression is explained by two hypotheses: dominance and overdominance. The dominance hypothesis attributes fitness declines to the expression of recessive or partially recessive deleterious alleles. These alleles persist due to the rapid removal of additive and dominant harmful mutations by selection. Conversely, the overdominance hypothesis posits that heterozygous genotypes have greater fitness than either homozygous alternative, leading to declines in fitness as homozygosity increases. Current evidence primarily supports the dominance hypothesis, suggesting that partially recessive deleterious mutations are the main drivers of inbreeding depression (Charlesworth and Willis, 2009).

The negative effects of increased selfing on offspring fitness may limit the evolution of selfing and favor outcrossing (Ozimec and Husband, 2011). Studies have demonstrated the impacts of inbreeding across various species. In *Cucurbita pepo ssp.*, inbreeding significantly affects vegetative vigor, germination percentage, and the time to first flower (Hayes et al., 2005). In *Camellia sinensis*, inbreeding dramatically reduces fruit production, with selfed offspring producing only 7.3% of the fruits observed in outcrossed progeny—a strong indicator of inbreeding depression (Liu et al., 2021). Similarly, in *Scabiosa columbaria*, severe inbreeding depression manifests in reduced biomass production, root development, adult survival, and seed set (Van Treuren et al., 1993).

In *Saxifraga granulata*, self-fertilization significantly reduced seed production and seed size by 23% and 48%, respectively, compared to outcrossed plants, highlighting the detrimental impact of selfing on seed traits (Walisch et al., 2012). Similarly, an investigation into *Silene vulgaris* revealed that self-fertilization significantly influenced seed viability and abortion rates fruit^{-1}. Self-pollination increased the number of viable seeds fruit^{-1} by 17% and reduced the number of aborted seeds fruit^{-1} by 90% compared to cross-pollination. However, the viable seeds from self-pollination were, on average, 4.5% lighter than those from cross-pollination (Glaettli and Goudet, 2006).

Inbreeding depression tends to intensify over successive generations of selfing due to the progressive increase in homozygosity. As homozygosity rises, there is a greater likelihood that deleterious recessive alleles will pair and express their effects. For example, in *Mimulus guttatus*, inbreeding depression in pollen production increased from 39% in the first generation to 80% by the fifth generation, while pollen viability exhibited a more than fourfold increase in inbreeding depression over the same period (Carr and Dudash, 1997).

In *Cucurbita moschata*, studies on inbreeding across four generations demonstrated a decline in seed yield and vigor. The most significant reductions occurred during the first generation of selfing, with subsequent generations showing a more gradual decrease. The results aligned with a linear regression model, suggesting that while fitness declines as homozygosity increases, the rate of decline slows as deleterious alleles are expressed and potentially purged from the population (Cardoso, 2004).

Self-fertilization is common in flowering plants and often results in inbreeding depression (Kariyat et al., 2021). In *Gerbera hybrida*, inbreeding causes a significant decline in performance, with a one-fourth reduction observed in each successive selfing generation (Huang et al., 1995). Similarly, a study by Saeidi et al. (2007) demonstrated notable inbreeding depression in the selfed progeny of *P. hybrida*, affecting traits such as plant height, flower diameter, flower number, plant vigor, percentage of wilted plants, and percentage of plants exhibiting chlorosis. However, certain traits, including the number of branches plant^{-1}, branch length, and plant diameter, remained unaffected.

Petunia flowers, known for their diverse colors, are among the most popular choices for gardens and green spaces, and they are widely cultivated across various regions of Iran. The primary method of cultivation is through seeds (Ghasemi-

Ghahsareh and Kafi, 2008), which entails significant annual costs for seed procurement, resulting in a substantial outflow of foreign currency. To address this, considerable efforts have been directed toward enabling seed production via self-pollination. However, it is crucial to note that the repeated use of self-pollinated seeds may alter both the quantity and quality of flowers and seeds over time (Saeidi et al., 2007a, b).

This study aimed to investigate the effects of self-pollination on various petunia genotypes over three generations. Additionally, flower color—a critical trait for flower identification and localization—has rarely been examined in the context of inbreeding (Garcia et al., 2019; Schrieber et al., 2021). This may be due to a prevailing focus on traits more directly associated with inbreeding depression, such as growth or reproductive success. Consequently, this study also explores how inbreeding influences levels of flavonoids and anthocyanins, providing valuable insights into the effects of self-pollination on flower pigmentation over three generations in petunia.

Materials and Methods

Plant materials

This study was conducted in a greenhouse at Ferdowsi University of Mashhad from 2021 to 2023, and examined the effects of forced self-pollination on trait performance of five petunia genotypes. Hybrid (F1) seeds of the following petunia genotypes were obtained: *P. hybrida* grandiflora Burgundy (P1) and *P. hybrida* grandiflora Blue (P2), sourced from Benary Seeds Co.; *P. hybrida* supercascade White (P3) and *P. hybrida* supercascade Red (P4), sourced from PanAmerican Seed Co.; and *P. hybrida* milliflora Rose Morn (P5), sourced from Syngenta Flowers Co. All seeds were procured through Sabz Royesh Company.

To ensure genetic diversity and significant differences in traits, F1 hybrids were selected to maximize heterozygosity. The F1 seeds of all genotypes were cultivated simultaneously in the greenhouse. Forced self-pollination was carried out for three successive growth seasons to produce the S1 generation (first generation resulting from self-pollination), the S2 generation (second generation resulting from self-pollination), and the S3 generation (third generation resulting from self-pollination). The procedure for self-pollination was as follows:

F1 Generation: After flowering, F1 plants were self-pollinated to produce S1 seeds. Self-pollination was conducted at the flower bud stage, precisely one day before anthesis. The anthers containing self-pollen (collected from a blooming flower on the same plant) were rubbed onto the stigma of the flower bud. Successful pollination was confirmed by the development of seed capsules, which matured approximately 3-4 weeks later, depending on the variety. The seeds were then collected and dried.

S1 Generation: The S1 seeds were sown, and the resulting plants were self-pollinated using the same method as described for the F1 generation. This process produced S2 seeds.

S2 Generation: The S2 seeds were sown, and the plants were again self-pollinated to produce S3 seeds, following the same procedure.

In each generation, the seeds obtained from self-pollination were cultivated to generate the next generation. The F1, S1, S2, and S3 seeds obtained from the five petunia genotypes constituted the genetic materials used in this study.

Experimental design and growth conditions

After obtaining the seeds, the experiment was conducted as a factorial experiment using a completely randomized design with five replications. Seeds from the five genotypes across all five generations were simultaneously cultivated in trays under greenhouse conditions, with average temperatures ranging from a maximum of 30 °C to a minimum of 20 °C. Once germinated, the seedlings were transplanted into pots filled with a substrate mixture of soil, sand, and perlite in a volumetric ratio of 2:1:1.

Assessment for morphological traits

The traits associated with each of the five genotypes (P1, P2, P3, P4, and P5) were measured in each of the 4 generations (F1, S1, S2, S3, and S4). These traits were the following:

Germination tests

Seeds from F1, S1, S2, and S3 obtained from five genotypes of petunia were sown on moist filter paper (Whatman No. 2) in a Petri dish with a diameter of 9 cm. Subsequently, the seeds were transferred to a germinator under 12 h light/12 h dark conditions at 25 °C and 70% relative humidity. From each genotype, 100 seeds (with five replications) were sown in each generation, with the criterion for germination being the emergence of a radicle at least 2 mm in length. After completing the germination period (14 d), the germination percent and mean germination time of each genotype in each generation were calculated using the following formula, where 'n' is the number of seeds newly germinated at time

'd,' and 'd' represents the number of days from the beginning of the germination test.

$$\text{Mean germination time} = \sum DN/N$$

$$\text{Germination percent} = \frac{\text{Number of germinated seeds}}{\text{Number of seed with viability}} \times 100\%$$

Height to first flower

Following the anthesis of the initial flower, the height was measured with a precision of 0.1 cm from the bed surface to the plant's highest point (Cao et al., 2019).

Flower diameter and corolla tube length

Five fully opened flowers were randomly selected from each replication to collect data on flower diameter and corolla tube length using a digital caliper. Flower diameter was measured across the petals from one edge to the opposite side, following the method described by Cao et al. (2019). Corolla tube length was measured from the base of the flower's calyx to the point where the petals curve, encompassing the entire tubular portion formed by the fused petals, as outlined by Teixeira et al. (2020).

Flower count

The number of flowers plant^{-1}, 21 d after the first flower opened, was counted weekly for 3 weeks, and the average was reported (Glaettli and Goudet, 2006).

Number of branches plant^{-1}

The number of branches was counted 21 d after the first flower opened.

Determination of total flavonoid and anthocyanin contents of petals

To ensure consistency in flower development, all flowers were harvested on the first day of anthesis. Two grams of flower petals were ground into powder using liquid nitrogen and dissolved in 3 mL of methanol containing 0.1% hydrochloric acid. The solution was then centrifuged, and the resulting supernatant was used to measure total flavonoid and anthocyanin content. A 300 µL aliquot of the solution was loaded into the wells of an ELISA plate reader (Synergy H11, BioTek), and absorbance spectra were recorded at 415 nm for total flavonoid content and 535 nm for total anthocyanin content, following the method described by Andersen and Jordheim (2010).

Statistical analysis

Statistical analyses of the experimental data were performed using Statistix 9.0 software, and mean comparisons for traits were conducted using the least significant difference (LSD) test at a 5% probability level.

Results

Flower diameter

The analysis of variance indicated that the effects of genotype and the genotype × inbreeding interaction on flower diameter were statistically significant at the 1% probability level, while the effect of inbreeding alone was significant at the 5% probability level (Table 1). Inbreeding led to a reduction in flower diameter in petunia, with the smallest flower diameter (52.68 mm) observed in the S3 generation, representing a 6.16% decrease compared to the F1 generation (Table 2). Flower diameter varied significantly among petunia genotypes, with P3 exhibiting the largest diameter and P5 the smallest. The interaction between genotype and inbreeding revealed that petunia genotypes responded differently to inbreeding across generations. Specifically, inbreeding reduced flower diameter in genotypes P2, P3, and P4, while it increased flower diameter in genotypes P1 and P5 (Table 2).

Table 1. Analysis of variance of different traits in *P. hybrida* genotypes P1, P2, P3, P4 and P5 across F1, S1, S2, and S3 generations.

S.O.V	Mean squares									
	DF	FlD	CTL	FlC	NBrPP	HFFl	TFlav	Acn	Gr%	MGrT
generation	3	53.38*	45.50**	239.87**	2.37^{ns}	23.69**	8.51**	16.98**	10280.00**	124.29**
genotype	4	2284.74**	694.86**	115.36**	15.41**	254.89**	627.43**	440.20**	3039.00**	7.22**
generation× genotype	12	140.63**	23.08**	5.82**	1.35^{ns}	6.24**	1.40*	1.43*	685.00**	3.57**
Error	80	14.56	5.46	0.93	0.96	1.57	0.67	0.74	92.50	0.71
CV (%)	-	7.05	7.58	14.36	20.94	19.75	8.25	10.89	14.31	9.14

S.O.V: source of variation, DF: degree of freedom, CV: coefficient of variance, FlD: flower diameter, CTL: corolla tube length, FlC: flower count, NBrPP: number of branches plant^{-1}, HFFl: height to first flower, TFlav: total flavonoid, Acn: anthocyanin, Gr%: germination percent, MGrT: mean germination time. *, **, significant at 0.05 and 0.01 probability levels, respectively, ^{ns} = non-significant.

Table 2. Comparison of average quantitative characters of flowers for 5 *P. hybrida* genotypes in F1, S1, S2 and S3 generations.

Genotype	Generation				Mean
	F1	S1	S2	S3	
	Flower diameter (Mm)				
P1	56.04^{e-i}	53.08^{g-i}	58.37^{c-f}	62.54^{cd}	57.51^{B}
P2	57.13^{e-g}	56.17^{e-h}	53.18^{g-i}	52.24^{hi}	54.68^{C}
P3	75.08^{a}	67.82^{b}	63.00^{c}	55.43^{f-i}	65.33^{A}
P4	60.71^{c-e}	58.00^{d-f}	56.63^{e-h}	51.28^{i}	56.66^{BC}
P5	31.72^{l}	35.24^{kl}	36.85^{k}	41.89^{j}	36.43^{D}
Mean	56.14^{A}	54.06^{AB}	53.61^{B}	52.68^{B}	
	Corolla tube length (Mm)				
P1	32.64^{cd}	31.78^{d}	35.09^{bc}	38.13^{a}	34.41^{B}
P2	26.01^{ef}	25.37^{e-g}	23.29^{f-h}	21.17^{h}	23.96^{C}
P3	34.98^{bc}	35.11^{bc}	37.12^{ab}	39.29^{a}	36.62^{A}
P4	31.97^{d}	32.00^{d}	35.12^{bc}	37.71^{ab}	34.20^{B}
P5	22.59^{gh}	25.02^{e-g}	26.09^{ef}	26.27^{e}	24.99^{C}
Mean	29.64^{B}	29.86^{B}	31.34^{A}	32.51^{A}	
	Flower count				
P1	8.25^{cd}	5.40^{f}	3.50^{gh}	2.30^{h-j}	4.86^{D}
P2	14.25^{a}	11.40^{b}	7.85^{cd}	6.05^{ef}	9.89^{A}
P3	13.25^{a}	11.00^{b}	6.15^{ef}	3.80^{g}	8.55^{B}
P4	8.50^{c}	5.25^{f}	2.25^{ij}	1.55^{j}	4.39^{D}
P5	7.55^{cd}	7.05^{de}	5.35^{f}	3.35^{g-i}	5.82^{C}
Mean	10.36^{A}	8.02^{B}	5.02^{C}	3.41^{D}	

For each trait in each column or row, each letter indicates a significance group, where values with the same letter are considered similar in significance, and different letters denote statistically significant differences between those values (LSD, $P < 0.05$).

Corolla tube length

The ANOVA results showed that the main effects of genotype, inbreeding, and their interaction on corolla tube length were statistically significant at $\alpha < 1\%$ (Table 1). Overall, inbreeding led to an increase in corolla tube length across petunia genotypes, with the highest increase observed in the S3 generation, representing a 9.68% increase compared to F1. The average corolla tube lengths for S1, S2, and S3 were all greater than the average for F1, indicating that self-pollination across these generations consistently increased corolla tube length. Among the genotypes, P3 exhibited the longest corolla tube, while P2 had the shortest (Table 2). Inbreeding resulted in an increase in corolla tube length for genotypes P1, P3, P4, and P5, whereas P2 showed a decrease. The most pronounced increase, a 17.95% rise in corolla tube length, occurred in P4 after three generations of self-pollination (Table 2).

Flower count

The analysis of variance revealed that genotype, inbreeding, and their interaction had a statistically significant effect on flower count at the 1% significance level (Table 1). Flower counts decreased progressively in the S1, S2, and S3 generations compared to F1, with considerable variation observed across generations. The largest reduction in flower count due to self-pollination occurred in the second generation for most genotypes, except for P5, where the greatest reduction was observed in the third generation (Table 2). Among the genotypes, P2 exhibited the highest flower count, while P4 had the lowest. Inbreeding consistently reduced flower counts in petunia, with the most pronounced depression observed in P4, which showed an 81.76% decrease after three generations of self-pollination that led to inbreeding (Table 2).

Number of branches plant^{-1}

The ANOVA results in this study showed that genotype significantly influenced the number of branches plant^{-1} at the 1% probability level. However, inbreeding and the interactive effects of inbreeding and genotype did not have a significant effect on this factor (Table 1). Genotypes P2 and P5 had the highest number of branches plant^{-1}, with no significant differences between them. The lowest number of branches plant^{-1} was observed in genotype P4, indicating diversity in branch counts in petunia (Table 3).

Height to first flower

The ANOVA results indicated that genotype, inbreeding, and their interaction had significant effects on the height to the first flower at $\alpha < 1\%$ (Table 1). Inbreeding led to an increase in the height to the first flower across the various petunia genotypes. The tallest height to the first flower (7.32 cm) was observed in the S3

generation, representing a 44.66% increase compared to F1 (Table 3). Stem height to the first flower varied among genotypes, with P3 exhibiting the tallest height and P2 the shortest (Table 3). The interaction between genotype and inbreeding showed that P3 experienced the most significant changes, with inbreeding causing a 77.68% increase in height to the first flower and a delay in flowering. In contrast, the least pronounced changes due to inbreeding were observed in P4 (Table 3).

Table 3. Comparison of average quantitative characters of stem for 5 *P. hybrida* genotypes in F1, S1, S2 and S3 generations.

Genotype	Generation				Mean
	F1	S1	S2	S3	
	Number of branches plant^{-1}				
P1	8.25^{cd}	5.40^{f}	3.50^{gh}	2.30^{h-j}	4.86^{D}
P2	14.25^{a}	11.40^{b}	7.85^{cd}	6.05^{ef}	9.89^{A}
P3	13.25^{a}	11.00^{b}	6.15^{ef}	3.80^{g}	8.55^{B}
P4	8.50^{c}	5.25^{f}	2.25^{ij}	1.55^{j}	4.39^{D}
P5	7.55^{cd}	7.05^{de}	5.35^{f}	3.35^{g-i}	5.82^{C}
Mean	10.36^{A}	8.02^{B}	5.02^{C}	3.41^{D}	
	Height to first flower (cm)				
P1	4.66^{d-g}	4.92^{d-f}	5.47^{de}	6.23^{d}	5.32^{B}
P2	3.18^{g}	3.55^{fg}	4.27^{e-g}	4.80^{d-f}	3.95^{C}
P3	8.56^{c}	12.80^{b}	14.09^{ab}	15.21^{a}	12.66^{A}
P4	4.56^{e-g}	4.76^{d-g}	4.92^{d-f}	5.00^{d-f}	4.81^{B}
P5	4.35^{e-g}	4.82^{d-f}	5.27^{de}	5.38^{de}	4.96^{B}
Mean	5.06^{C}	6.17^{B}	6.80^{AB}	7.32^{A}	

For each trait in each column or row, each letter indicates a significance group, where values with the same letter are considered similar in significance, and different letters denote statistically significant differences between those values (LSD, $P < 0.05$).

Total flavonoid

The ANOVA results demonstrated significant effects of genotype ($\alpha < 1\%$), inbreeding ($\alpha < 1\%$), and their interaction ($\alpha < 5\%$) on total flavonoid content (Table 1). Inbreeding in the S3 generation led to a significant decrease in total flavonoid content, while no significant differences were observed between S1, S2, and F1 (Table 4). Among the genotypes, P4 had the highest total flavonoid content (14.89), whereas P3 exhibited the lowest (0.88). The interaction between genotype and inbreeding revealed that inbreeding reduced total flavonoid levels in P1, P2, and P5, with the most pronounced decreases occurring in the S3 generation. Compared to F1, total flavonoid levels in these genotypes decreased by 8.31%, 26.25%, and 11.39%, respectively (Table 4). However, inbreeding had no significant impact on total flavonoid levels in P3 and P4.

Table 4. Comparison of average qualitative characteristics of flowers for 5 *P. hybrida* genotypes in F1, S1, S2 and S3 generations.

Genotype	Generation				Mean
	F1	S1	S2	S3	
	Total flavonoid (µg mL^{-1})				
P1	14.32^{ab}	14.24^{ab}	13.86^{bc}	13.13^{cd}	13.89^{B}
P2	12.42^{de}	11.90^{e}	11.71^{e}	9.16^{f}	11.30^{C}
P3	0.91^{h}	0.89^{h}	0.87^{h}	0.83^{h}	0.88^{E}
P4	15.15^{a}	15.12^{a}	15.05^{a}	14.26^{ab}	14.89^{A}
P5	9.13^{f}	9.10^{fg}	8.79^{fg}	8.09^{g}	8.78^{D}
Mean	10.38^{A}	10.25^{A}	10.06^{A}	9.10^{B}	
	Anthocyanin (µg mL^{-1})				
P1	12.17^{ab}	11.78^{a-c}	10.98^{cd}	9.94^{d}	11.21^{A}
P2	11.46^{a-c}	11.11^{bc}	10.00^{d}	8.36^{e}	10.24^{B}
P3	0.55^{h}	0.56^{h}	0.50^{h}	0.45^{h}	0.51^{D}
P4	12.51^{a}	12.12^{ab}	11.53^{a-c}	9.93^{d}	11.52^{A}
P5	6.55^{f}	6.16^{fg}	5.80^{fg}	5.20^{g}	5.93^{C}
Mean	8.65^{A}	8.35^{A}	7.76^{B}	6.78^{C}	

For each trait in each column or row, each letter indicates a significance group, where values with the same letter are considered similar in significance, and different letters denote statistically significant differences between those values (LSD, $P < 0.05$).

Anthocyanin

The ANOVA results indicated that genotype and inbreeding significantly influenced anthocyanin levels at a 1% probability level, while their interaction was significant at a 5% probability level (Table 1). Inbreeding led to a reduction in anthocyanin levels in petunia, with the most substantial decrease (21.62%) observed in the S3 generation compared to F1 (Table 4). Among the genotypes, P1 and P4 exhibited the highest anthocyanin levels, with no significant difference between them (Table 4). The interaction analysis showed that P2 experienced the most pronounced reduction in anthocyanin levels due to inbreeding, with a 27.05% decrease compared to its respective F1. Conversely, inbreeding had no significant effect on anthocyanin levels in P3. The third generation of inbreeding (S3) caused the most notable changes in anthocyanin content, highlighting it as the generation with the greatest reduction (Table 4).

Germination percentage

The ANOVA results revealed that genotype, inbreeding, and their interaction had statistically significant effects on germination percentage at the 1% significance level ($\alpha < 1\%$) (Table 1). Increasing generations of inbreeding in petunia led to a significant decline in germination percentage, with the largest reduction observed in the S3 generation, amounting to a 48.20% decrease compared to F1 (Table 5). Among the genotypes, P2 exhibited the highest germination percentage, which was not significantly different from P5 (Table 5). In contrast, P3 showed the lowest germination percentage after three generations of self-pollination. Furthermore, P3 experienced the most dramatic changes in germination percentage within a single generation, with a 57.14% reduction in S2 compared to S1 (Table 5).

Table 5. Comparison of average seed germination traits regarding five *P. hybrida* genotypes in F1, S1, S2, and S3 generations.

Genotype	Generation				Mean
	F1	S1	S2	S3	
	Germination percent				
P1	88.00^{a}	80.00^{ab}	40.00^{cd}	30.00^{de}	59.50^{B}
P2	90.00^{a}	88.00^{a}	80.00^{ab}	70.00^{b}	82.00^{A}
P3	90.00^{a}	70.00^{b}	30.00^{de}	20.00^{e}	52.50^{C}
P4	88.00^{a}	80.00^{ab}	50.00^{c}	40.00^{cd}	64.50^{B}
P5	88.00^{a}	80.00^{ab}	72.00^{b}	70.00^{b}	77.50^{A}
Mean	88.80^{A}	79.60^{B}	54.40^{C}	46.00^{D}	
	Mean germination time (d)				
P1	7.57^{hi}	8.34^{h}	11.21^{b-d}	12.21^{ab}	9.84^{A}
P2	6.66^{ij}	6.74^{ij}	9.88^{ef}	10.09^{ef}	8.34^{D}
P3	6.78^{ij}	7.00^{ij}	11.90^{b}	13.00^{a}	9.67^{AB}
P4	6.47^{j}	6.74^{ij}	10.68^{de}	11.74^{bc}	8.91^{C}
P5	8.19^{h}	8.49^{gh}	9.45^{fg}	10.80^{c-e}	9.23^{BC}
Mean	7.14^{C}	7.46^{C}	10.63^{B}	11.57^{A}	

For each trait in each column or row, each letter indicates a significance group, where values with the same letter are considered similar in significance, and different letters denote statistically significant differences between those values (LSD, $P < 0.05$).

Mean germination time

The ANOVA results indicated that genotype, inbreeding, and their interaction significantly affected the mean germination time at the 1% level (Table 1). Inbreeding led to a notable increase in mean germination time in petunia, with the greatest increase observed in the S3 generation, representing a 62.04% rise compared to F1 (Table 5). The shortest mean germination time was recorded in P2. The interaction between genotype and inbreeding showed that although the first generation of self-pollination did not significantly affect mean germination time, subsequent generations of inbreeding led to substantial increases. Notably, after three generations of self-pollination, mean germination time in P3 increased by 91.74% compared to F1, demonstrating a strong inbreeding effect on this genotype. In contrast, P5 exhibited the smallest change, with a 31.87% increase in mean germination time in S3 compared to F1 (Table 5).

Discussion

The impact of inbreeding depression on flowering plants, which negatively affects traits related to reproductive success and survival, has been well-documented (Kariyat et al., 2021). Inbreeding is often associated with reductions in flower size and flower number (Glaettli and Goudet, 2006). For example, in this study, increasing generations

of inbreeding led to significant decreases in flower diameter in genotypes P2, P3, and P4. In the S3 generation, flower diameters in these genotypes were reduced by 8.56%, 26.17%, and 15.53%, respectively, compared to their F1 generation. These findings align with previous research; for instance, Kariyat et al. (2021) reported an 8.7% reduction in flower size after one generation of selfing in *Solanum carolinense*. Similarly, reductions in flower diameter due to inbreeding have been observed in *Petunia hybrida*, *Crepis tectorum*, *Foeniculum vulgare*, and female flowers of *Silene latifolia* (Andersson, 2012; Saeidi et al., 2007; Schrieber et al., 2021; Shojaiefar et al., 2022).

Among genotypes that experienced reductions in flower diameter due to inbreeding (P1, P2, and P3), the largest decrease occurred in the first selfing generation, with subsequent generations showing a more gradual decline (Table 2). This pattern aligns with findings in *Silene vulgaris*, where the most significant reduction in flower diameter was observed in the first selfing generation (Glaettli and Goudet, 2006). The diminishing intensity of inbreeding depression in later generations has been attributed to genetic load purging, the fixation of deleterious alleles through drift, or adaptation to greenhouse conditions (Barrett and Charlesworth, 1991; Glaettli and Goudet, 2006; Groom and Preuninger, 2000).

Interestingly, inbreeding led to an increase in flower diameter in genotypes P1 and P5, illustrating the diverse responses of different petunia genotypes to inbreeding (Table 2). In genotype P1, flower diameter decreased in S1 compared to the parents but increased in S2, suggesting that the second generation of selfing may have disrupted certain gene linkages, resulting in increased flower diameter. These results highlight significant differences among genotypes in their responses to inbreeding, reflecting their varying sensitivities.

Specifically, comparisons between genotypes P2 and P3 show contrasting patterns. Based on the averages of S1, S2, and S3, genotype P2 exhibited greater stability, with less pronounced reductions in flower diameter. Conversely, genotype P3 was strongly affected by inbreeding, as evidenced by substantial decreases in flower diameter across its S1, S2, and S3 generations compared to its F1 generation.

Inbreeding led to an increase in the corolla tube length in petunia genotypes, likely driven by additive gene action (Table 2). According to van Ginkel and Ortiz (2018), during inbreeding and selfing, co-adapted gene complexes are selected and fixed in superior plants within a population. The resulting superiority of inbred progeny is often attributed to additive gene action and the epistatic effects of additive-by-additive interactions. However, repeated inbreeding can lead to a monotonic increase in inbreeding depression, limiting the favorability of selfing variants due to the heightened homogeneity caused by close-relative mating or selfing (Ozimec and Husband, 2011). One factor constraining the evolution of selfing and favoring mixed-mating systems is the negative correlation between selfing rates and the fitness of selfed offspring (Goodwillie et al., 2005).

In *Silene latifolia*, for instance, inbred lines exhibited smaller flowers with altered spatial arrangements (Schrieber et al., 2021). Similarly, in the present study, inbreeding resulted in both an increase in corolla tube length and a decrease in flower diameter, causing notable morphological changes in petunia flowers. Furthermore, an increase in inbreeding generations significantly reduced flower numbers in petunia (Table 2). These results align with findings by Ozimec and Husband (2011), who reported a positive relationship between inbreeding depression and the number of inbreeding generations.

In gerbera, inbreeding depression was observed in cut-flower yield, with a 1% increase in inbreeding per generation reducing yield by one-quarter flower per generation (Huang et al., 1995). Recurrent self-pollination in octoploid strawberries led to yield reductions of 57% and 80% in the S2 and S5 generations, respectively (Aalders and Craig, 1968). Such findings highlight that inbreeding depression often increases monotonically with successive selfing generations due to the cumulative decline in heterozygosity (Ozimec and Husband, 2011).

Similar patterns have been observed in *Mimulus guttatus*, where selfed progeny showed a gradual decline in performance across five generations, with inbreeding depression intensifying over time (Carr and Dudash, 1997). In cyclamen, Wellensiek (1959) demonstrated that self-pollination over five consecutive generations resulted in a marked decline in flower numbers, decreasing from 34.0 in the first generation to 15.3 in the fifth. These negative effects are likely due to numerous slightly deleterious recessive alleles, which are challenging to purge (Husband and Schemske, 1996).

Walisch et al. (2012) also reported significant inbreeding depression in flower counts in the second generation of *Saxifraga granulata*. They concluded that a single generation of inbreeding was insufficient to reduce the genetic load, consistent with the findings of this study.

Similarly, in *Chamerion angustifolium*, Ozimec and Husband (2011) found cumulative inbreeding depression increasing over three generations. They noted that the purging of deleterious mutations could not keep pace with rising homozygosity, likely due to the combined effects of numerous deleterious alleles, which are difficult to purge (Carr and Dudash, 1997).

Inbreeding led to an increase in the height to the first flower in petunia. Since stem height is positively correlated with flowering time, this increase suggests that inbreeding delays flowering in petunia. Similarly, a study on inbreeding in *Silene vulgaris* reported a delay in flowering, with self-pollinated offspring taking 96.7 days to flower compared to 89.1 days for cross-pollinated offspring (Glaettli and Goudet, 2006).

Additionally, the findings of Saeidi et al. (2007) showed that self-pollination did not significantly affect the number of branches plant^{-1} in *petunia*, consistent with the current study. The lack of significant differences between S1, S2, S3, and F1 generations in petunia may be attributed to the limited role of dominant genetic effects on the number of branches.

Flower color is a complex trait influenced by chemical interactions between two flavonoid types: anthocyanins and copigments (Hondo et al., 1992). In *Silene latifolia*, inbreeding did not affect flower color after one generation of self-pollination (Schrieber et al., 2021). Conversely, in octoploid strawberries, self-pollination in the S1 generation caused a 21% reduction in fruit color, with further reductions of up to 80% observed in subsequent generations after S2 (Aalders and Craig, 1968). In another study, inbreeding resulted in strawberries with lighter-colored fruits and surface achenes (Rho et al., 2012).

In the present study, inbreeding in the S3 generation significantly reduced total flavonoid and anthocyanin contents, indicating that multiple self-pollination generations are required to observe these effects. Anthocyanins are key pigments that contribute to flower petal color, and most color changes in plants are associated with anthocyanin variations (Lippi et al., 2011). Consistent with this, *Silene vulgaris* selfed offspring produced less anthocyanin than crossed offspring (Sandner and Matthies, 2018). This general reduction in anthocyanin levels with inbreeding has been suggested to occur due to a disruption in one of the steps in the red pigment synthesis pathway or because selfed plants may not afford the metabolic costs of anthocyanin production (Misyura et al., 2013; Sandner and Matthies, 2018).

In contrast, *Camellia sinensis* exhibited increased leaf anthocyanin content as a result of inbreeding (Liu et al., 2021). Similarly, in *Mimulus parishii*, self-pollination led to reduced anthocyanin concentration in petals, which was attributed to a causal mutation in the 5′ untranslated region that introduced an upstream ATG start codon. This mutation decreased the translation efficiency of the R2R3-MYB gene, attenuating protein translation and resulting in reduced flower coloration in self-pollinating species (Nelson et al., 2021; in Liang et al., 2022). In *petunia*, the R2R3-MYB transcription factor is associated with the complex patterns of petal pigmentation (Albert et al., 2014). The reduction in anthocyanin levels observed in this study may be explained by weakened protein translation, potentially caused by similar mechanisms as those observed in *Mimulus parishii*.

Inbreeding led to a reduction in germination percent in petunia (Table 5). This reduction can be attributed to increased homogeneity in offspring, which diminishes genetic diversity within the population and increases the expression of deleterious alleles. These factors often result in weaker offspring, reducing their chances of survival (Charlesworth and Willis, 2009; Sandner and Matthies, 2018). Similar negative effects of inbreeding on seed germination have been reported in strawberries and *Saxifraga granulata*, where germination rates decreased by 83.2% and 20%, respectively, due to inbreeding (Kaczmarska et al., 2014; Walisch et al., 2012).

In this study, the lowest germination percent after three generations of self-pollination was observed in genotype P3, which exhibited a dramatic 77.77% reduction compared to F1. This indicates that P3 is particularly sensitive to inbreeding. In contrast, genotypes P2 and P5 displayed greater stability in germination percent across S1, S2, and S3 generations, suggesting they are less affected by inbreeding (Table 5).

The reduction in vigor observed with inbreeding depression is primarily due to the homozygosity of slightly deleterious recessive alleles (Labroo et al., 2023; Liu et al., 2021). Additionally, inbreeding resulted in an increase in the mean germination time in petunia (Table 5). This trend has also been noted in *Silene vulgaris*, where self-pollinated seeds took an average of 13.1 days to germinate compared to 10.8 days for outcrossed seeds (Glaettli and Goudet, 2006). Similarly, Dudash et al. (1997) observed in *Mimulus guttatus* that serial inbreeding increased germination time and negatively impacted other fitness traits, such as flower production and the

timing of first flowering, with little evidence for the purging of deleterious alleles.

Conclusions

The level of inbreeding depression increased with successive generations of self-pollination in *petunia*. This increase was particularly evident in traits such as flower production, which showed the greatest sensitivity to inbreeding. The relative reduction in flower count, a complex trait with relatively low heritability, was 67.08% from F1 to S3, highlighting its significant vulnerability to the effects of homozygosity. In contrast, traits such as the number of branches per plant were less affected by inbreeding and exhibited greater stability across generations. Anthocyanin, an essential pigment in petunia, exhibited a 21.62% reduction due to inbreeding. The generational analysis revealed that traits such as flower count and germination percent experienced the most significant reductions in the second generation of inbreeding, while total flavonoid and anthocyanin content showed the highest reductions in the third generation. Overall, self-pollination in petunia increases homogeneity within the population, resulting in the expression of undesirable recessive alleles previously masked by dominant alleles. Consequently, this leads to a reduction in the performance of several traits due to the cumulative effects of inbreeding depression.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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