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POLLINATION, SEED SET AND POLLEN TUBE GROWTH INVESTIGATIONS IN VIOLA PEDATA L.

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Wayne Alan Becker

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Harold C. Evans

Major professor

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POLLINATION, SEED SET AND POLLEN TUBE GROWTH INVESTIGATIONS IN *VIOLA PEDATA* L.

By

Wayne Alan Becker

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ABSTRACT

POLLINATION, SEED SET AND POLLEN TUBE GROWTH INVESTIGATIONS IN VIOLA PEDATA L.

By

Wayne Alan Becker

Plants of Viola pedata collected from the wild in Michigan, Arkansas, Tennessee and Wisconsin, produced very low amounts of seed when grown in a greenhouse and self-pollinated. The objectives of these investigations were: 1) to determine the reason(s) for low (self) seed set and; 2) to determine how seed set could be increased.

Pollen viability measured by cotton blue staining, pollination and seed set studies, and pollen and pollen tube observations using fluorescence microscopy were employed. Cross-pollinations resulted in significantly greater quantities of seed than did self-pollinations, and many fewer pollen tubes gained entry to the ovaries in self-pollinated pistils compared to cross-pollinated pistils.

These investigations indicate that a self-incompatibilty system is operative in Viola pedata, that this is responsible for the low (self) seed set values, and that cross-pollination can increase seed set.

DEDICATION

This work is dedicated to the memory of my mentor and beloved grandfather, Gilbert J. Hoehn. He brought great joy to his family and friends, made the best wild strawberry ice cream, and was the horticultural envy of Ottoville, Ohio.

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I wish to extend my appreciation to the people who so kindly assisted in this research and manuscript preparation. To Dr. Lowell Ewart, a most patient major professor, for introducing me to *Viola pedata*, and for allowing me to investigate its wonders. To my committee members, Dr. Joanne Whallon and Dr. Art Cameron, and to my colleagues, Candice Shoemaker and Steve Krebs, for their ideas, advice, and friendship. To my loving families, for their support and understanding.

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Introduction

Viola pedata L., the birdfoot violet, is a perennial wildflower native to the eastern half of the United States. Its aesthetic qualities indicate that it could become a more widely utilized garden plant and/or be developed into a pot plant. Presently it is sold as a vegetatively propagated crop through specialty nurseries, which may propagate the plants in-house and/or rely on plants collected from the wild. Inconsistent availability is frequently a problem. It would be of benefit to the ornamental horticulture industry and to the gardening public if seed-produced cultivars could be developed. In addition, *V. pedata* is listed as endangered in several states. A greater knowledge of its reproductive biology would aid in its preservation and reestablishment.

Preliminary attempts to produce (self) seed from a single specimen of *V. pedata* collected in Michigan, and from a group of plants collected in Arkansas, met with low, inconsistent seed set. Such low seed yields would hinder breeding efforts and commercial seed production.

This thesis reports initial investigations into the sexual reproduction of *V. pedata*, centering on pollination, pollen tube and seed set studies. The objectives of this

research were 1) to determine the reason(s) for low (self) seed set and 2) to determine how seed set could be increased.

Literature Review

Viola pedata, the birdfoot violet, is a member of the Violaceae family, section Nominium, sub-section Plagiostigma and group Pedatae (22). It is described as monotypic, very distinct and without any close relatives (30). Distinguishing characteristics [e.g. the massive style and its unique form, a complete absence of cleistogamous flowers, the presence of adventitious buds on the roots, and a unique chromosome number of 2N=56 (16)] distance V. pedata from other members of the Plagiostigma sub-section. Gershoy notes that, phylogenetically, V. pedata is probably most closely related to the species of the Boreali-Americanae and Adnatae groups (22).

The morphology of V. pedata has been described by a number of authors (8, 13, 31). The following is a compilation of their descriptions, along with observations made by the author.

V. pedata can be found growing in sunny areas on sandy, well-drained, acidic soils. It is a stemless blue violet with a rosette of leaves growing from a short, thick, vertical rhizome. The leaves are deeply cut and palmately divided into 3-5 sections, each section being cleft or toothed near the apex. V. pedata is one of the most polymorphic violets and many additional names have been

assigned to the various forms. Different leaf forms may be found on the same plant at the same time and/or at different times of the year (31).

The flowers of V. pedata are stalked, solitary and nodding, with a diameter of 2-4 cm (Figure 1). The calyx consists of five sepals and the corolla of five petals. Four of the five petals are arranged in two pairs, each pair differing in shape. The fifth, lower petal is spurred and beardless. There are two principle types of coloration. The concolor type has all five petals one shade of blue (Figures 2, 3). In the bicolor type, the upper two petals are a dark purple and the lower three are blue (Figure 1). Thin, dark-purple markings may pattern any or all of the petals, appearing as lines originating at the base of the petal(s).

The five stamens have very short filaments and form a tube enclosing the ovary and part of the style. Orange connective tissue extends from the distal end of the anthers and closes tightly around the style.

The pollen is shed on the inside of the staminal tube, falls into the groove of the lowermost petal and is retained there. This groove leads to the spur of the petal containing the nectar. The two nectaries are appendages of the two lowermost stamina.

The stigmatic surface is cupped, with a lip on the lower side, and ringed with papillae. The style is hollow, with a very flexible hinged area just above the ovary. The ovary is one-celled with many ovules.

Figure 1. Viola pedata plant with bicolor flowers.

Figure 2. Concolor flower with narrow petals.

Figure 3. Concolor flower with wide petals.

Figure 4. Fruit capsule 25 days after pollination.

The fruit is an ellisoid capsule and dehisces along three sutures (Figures 4, 5). The seed are light to dark brown, smooth, round to ellipsoid, and approximately 1 mm in diameter (Figure 6). The seed are forcibly ejected from the dehisced capsule.

Beattie and Culver stated that the flowers of V. pedata failed to set seed in the abscence of insect visitors in field and greenhouse trials (7). Beattie described V. pedata as a self-compatible species and stated that insect pollination resulted in varying degrees of self- and cross-pollination depending on the type of insect, plant spacing, the number of flowers present on individual plants, and other factors (6).

Gershoy reported that "the capacity for self-fertilization is retained for at least five days in many (Viola) species, as determined by hand-pollination of old blossoms" (22). Kristofferson reported that thrips pollinated V. tricolor flowers as a result of living in the spur petals and the stigmatic cavity (26).

In a study of approximately 75 Viola species, including V. pedata, Gershoy observed that, upon artificial self-pollination, the pollen tubes reached the upper portion of the ovary cavity, through the hollow style, within 14 to 16 hours. Gershoy also mentions that the species used in these studies were grown for several generations using self seed (21).

There is no consistent evidence of the occurrence of

Figure 5. Dehisced fruit from which several seed have been ejected.

Figure 6. Seed from fruit shown in Figure 5.

self-incompatibility in the violets (Viola sp.) (21, 22, 37). However, Emino, investigating low seed set among twenty-nine inbred breeding lines of V. tricolor Hortensis L., reported the possible existence of a self-incompatability system (19). According to Emino, cross-pollinations usually resulted in greater seed set than did self-pollinations, especially when low self seed set types were used as male parents in crosses onto other lines. Emino stated that the increased seed set in cross pollinations was not consistent (i.e. for some inbred lines, cross-pollinations with certain other lines did not result in higher seed set compared to the self seed set of one and/or both of lines involved) and that the problem of low seed set is therefore complex in nature. Pollen fertility, differing chromosome numbers, and meiotic division did not appear to contribute to low seed set among the inbred lines studied.

Materials and Methods

Plant Material and Culture

The *V. pedata* plants were obtained from four geographic areas. One concolor plant was collected in Michigan (MI group). Nearly one hundred were collected by the author in March of 1986 in the vicinity of Hot Springs Village, Arkansas (AR group). They were very heterogeneous in appearance, approximately half being concolors and half bicolors. Six concolor plants were obtained from a nursery in Nebraska, but had originally been collected in Tennessee (TN group). Their genetic relationship is unknown. One hundred plants (50 bicolor, 50 concolor) were purchased from a nursery in Wisconsin (WI group), were native to that state, and were more uniform in appearance than the Arkansas plants. Plants of the WI group had been propagated by the nursery both sexually and asexually so that they represented a number of genotypes and their clones, and reportedly set seed regularly when grown out in beds (Personal Communication: Prairie Nursery, Westfield, WI).

The F1 plants were grown from seed produced from cross-pollinations between and among plants of the MI and AR groups. No guidelines for the germination of *V. pedata* seed were found in the literature. Attempts to germinate both self and cross seed were made using recommendations for

other Viola species including such treatments as 0.2% KNO_3, 100-400 ppm GA_3, light, pre-chill and scarification (1, 3, 18).

All plants were propagated asexually by division when possible to increase the number plants to provide more flowers for pollinations. Each plant and its clones were given the same identification number.

Since the literature was void of greenhouse cultural requirements for V. pedata, the following procedures were utilized. The plants were potted up in 4 or 6 inch clay pots, depending on the size of the plant. A soilless mix (2 peat : 1 coarse sand : 1 pea gravel) was used, along with an inch of pea gravel in the bottom of the pot for adequate drainage. The plants were kept in the greenhouse on raised benches. In the winter the greenhouse temperature was set at 20°C, day and night. In the summer, a fan and pad cooling system was used to keep the greenhouse as cool as possible during the day. Night temperatures were allowed to drop as low as 10° C during the spring, summer and fall. Supplemental lighting (high pressure sodium vapor) was applied from November to March of 1986-87 to keep the daylength at a minimum of 10 hours in order to encourage growth and flowering. The plants were fertilized every three months with a 300 ppm 20-20-20 soluble fertilizer. Iron sulfate was applied to keep the growing medium at a pH of 3 to 4. A fungicide (Benlate) was applied to control Rhizoctonia spp. root and stem rot, and the plants were

watered only when the top inch of growing medium was dry. Pesticides (Temik and Metasystox-R) were applied as recommended to control thrips and red spider mites. Flowers which bloomed within two days after pesticide spraying were not used.

Experimental Methods

The availability of flowers on plants from the various groups determined the extent of each group's involvement in the following experiments.

Experiment 1. Anther dehiscence. To determine when anther dehiscence occurred, observations in a different set of 5 flowers each from of the AR and WI groups were made at each of 4 stages of flower development: 1.) petals still closed, color well-defined, flower just about to open; 2.) petals open approximately halfway; 3). anthesis; 4.) one day after anthesis. The number and position(s) of dehisced anthers was recorded.

Experiment 2. Pollen viability. To determine whether poor pollen viability was a factor contributing to low (self) seed set, 100 pollen grains each from 3 flowers per plant, from twelve different plants, were scored for viability using cotton blue stain (equal parts phenol crystals, lactic acid, glycerine and distilled water) (24). Plants were chosen which represented a range of average (self) seed set values.

A mixture of pollen from all five anthers of each flower was collected on the day of anthesis. A quantity of pollen was transferred onto a drop of stain on a slide, and a coverslip was placed over the drop. Observations were made after 10 minutes, using a compound microscope (Olympus model BHS) at 10X. The pollen grains were scored either as plump and darkly-stained (indicating viability) or as misshapen and lightly-stained.

Experiments 3a-g. Seed set. The following pollination and seed set measurement techniques were used in all seed set experiments.

Pollination. For self-pollinations, pollen from the anthers and/or groove of the spur petal was transferred to the stigma with a blunt needle probe. The pollen was placed into the stigmatic cavity and around its perimeter.

Cross-pollinations were made in a similar manner. Using a fine forceps, the female parents were emasculated prior to anthesis by either removing the spur petal and anthers (Experiment 3b), or the spur petal alone (Experiment 3f).

Each flower was pollinated only once. The flowers were tagged and pollinated either on the day of anthesis or one day after anthesis unless otherwise indicated. All pollinations were made in the greenhouse which was equipped with screens to exclude insects which might effect extraneous pollinations.

Seed Set Measurement. The flowers were scored for seed

capsule formation two weeks after pollination. Four weeks after pollination, the capsules were collected, allowed to dry at room temperature for aproximately one week, and the seeds then counted.

Experiment 3a. To determine the extent of seed set on flowers which were not artificially pollinated, observation were made of 100 flowers each from the AR and WI groups, and 20 each from the TN and MI groups. The flowers were tagged as buds and allowed to mature without further disturbance until being scored for seed capsule formation.

Experiment 3b. To determine if agammospermy could be responsible for any seed set, and to evaluate the efficacy of the emasculation technique, 50 flowers each from the AR and WI groups were emasculated, as buds, by removing the spur petal and the anthers. The emasculted flowers were covered with small glycine bags to prevent extraneous pollination.

Experiment 3c. To determine the effect of flower age at pollination on seed set, 40 flowers each from the AR and WI groups were self-pollinated on one of the five days beginning at anthesis. The pollinations for each day were made between 10 am and 12 noon.

Experiment 3d. To determine when stigmatic fluid exuded and to determine the effect of stigmatic fluid on seed set, the following two methods were used. (Stigmas on which a drop

of stigmatic fluid could be seen are referred to as "wet"; "dry" stigmas had no visible stigmatic fluid.)

Experiment 3d(i). Fifty flowers each from the AR and WI groups were self-pollinated at each 10 am and 3 pm on the day of anthesis. A record was kept of whether or not the stigma was wet when pollinated.

Experiment 3d(ii). Fifty flowers each from the AR and WI groups were observed at 10:00 am and 3:00 pm on the five days beginning at anthesis. If the stigma was wet, the flower was self-pollinated. Flowers were not pollinated if their stigmas remained dry for the five days.

Experiment 3e. To evaluate the self-compatability of each of the plants in all groups, 10 flowers per plant were self-pollinated. The average seed set per pollination was used as a measure of each plant's self-compatibility.

Experiment 3f. To evaluate the cross-compatibility of each plant, and to compare cross vs self seed set, random cross-pollinations were made among and between many of the plants from all four groups. Each plant was crossed separately with each of at least 3 different male parents. The average seed set per pollination was calculated from the total seed produced by at least 3 flowers per plant, and was used as a measure of each plant's cross-compatibility.

Experiment 3g. To evaluate the self-compatability of the F1 plants, and to determine likelyhood of inbreeding depression as a factor contributing to low self seed set of the

parental plants, up to 10 flowers from each of the F1 plants were self-pollinated. The average seed set per pollination was used as a measure of each plant's self-compatibility.

Experiment 4a,b. Fluorescence microscopy investigations.

Experiment 4a(i). To observe pollen and pollen tube growth in pistils of flowers from plants of all groups, the flowers were either self- or cross-pollinated and then harvested after 24, 48, 72, 96, 120 or 144 hours. Fluorescence microscopy, from a method described by Kho and Baer with some modification, was used to observe pollen and pollen tubes in the pistils of the harvested flowers (25). This procedure is based on the fact that the pollen tubes contain callose, a material which selectively takes up aniline and consequently fluoresces when illuminated by blue or ultraviolet light. With this technique, the pollen tubes fluoresce bright green against a dark background, especially under ultraviolet light.

The pistils were carefully dissected from the flowers and placed in 8N NaOH to clear and soften. After 24 hours, the pistils were rinsed in distilled water for 6 to 24 hours. The pistils were then transferred to a solution of 0.1% aniline w.s. dissolved in 0.1 N K_3PO_4 for at least 15 minutes, or until the stain had thoroughly penetrated the pistil. A stained pistil was placed on a slide in a drop of stain, and then split open longitudinaly, using a thin, sharp razor. The two halves were laid out so that the cut

surfaces faced upward.

Observations were made under a binocular microscope (Olympus model BHS) equipped with a high pressure mercury vapor lamp. The lamp, along with the proper blue and UV barriers and filters (Olympus L-420, 0515), provided the required light waves between 350 and 400 mu. Using a 2X objective, the pistil was examined for pollen quantity and germination, pollen tube growth and probable fertilizations. A coverslip was then placed over the pistil and slight pressure applied. Further observations were then made under higher magnifications (10 and 20X).

The quantity of pollen in the stigmatic cavity of each pistil was categorized as: 0.) no pollen; 1.) one to approximately 100 pollen; and 2.) approximately 100+ pollen. The quantity of germinated pollen was categorized as 0.) no germinated pollen; 1.) one to approximately 100 germinated pollen; and 2.) approximately 100+ germinated pollen.

Pollen tube growth in each pistil was categorized as: 0.) no pollen tubes or tubes shorter than diameter of pollen; 1.) tubes within the style; 2.) tubes at the bottom of the style/top of the ovary; 3.) tubes in the ovary; 4.) tubes penetrate ovules. The number of probable fertilizations per pistil was determined by counting the number of ovules into which pollen tubes had penetrated.

Experiment 4a(ii). To observe pollen and pollen tube growth in pistils of the F1 plants, flowers were selfed, crossed or back-crossed and harvested after 144 hours. Observations

were made using the fluorescence microscopy technique described above for Experiment 4a(i).

Experiment 4b. To estimate potential seed set, ovule counts were obtained from many of the specimens prepared in Experiment 4a. The average number of ovules per ovary for each plant was used as a measure of its seed set potential. The averages were calculated from counts of the total number of ovules in at least 3 different ovaries per plant.

Statistical Analyses. The MSTAT Microcomputer Statistical Program (MSTAT Development Team, 1986) was used for all statistical analyses. The "Manager", "Sort", and "AOV" (ANOVA-1) programs were utilized.

Results

Experiment 1. Anther dehiscence. There was little observable difference between the AR and WI groups regarding anther dehiscence (Table 1). Dehiscence usually began before the flower opened (Stage 1; tight bud showing color) and was completed one day after anthesis (Stage 4). Flowers went through the 4 stages of development in 2-3 days. The anthers dehisced in a specific order, beginning with the anther situated between the upper pair of petals. Next, the two anthers adjacent to this upper anther would dehisce, followed by the lower two anthers.

Experiment 2. Pollen Viability. Pollen viability as determined by cotton blue staining was high in all tested plants. Average counts ranged from 75 to 97 plump, darkly-stained pollen out of 100 (Table 2). One pollen grain in Figure 7 appears shrunken and lightly-stained, and is typical of grains scored as non-viable; the pollen scored as viable appear round, plump and darkly-stained.

Experiments 3a-g. Seed set.

Experiment 3a. For flowers which were not artificially pollinated, the average seed set for all groups combined was

Table 1. Anther dehiscence in flowers of Arkansas (AR) and Wisconsin (WI) plants.

Stage of Flower Development	Ave. No. of Dehisced Anthers[1]		
	AR	WI	Combined
1. Tight bud/color apparent	1.2	0.6	0.9
2. Open halfway	1.4	1.4	1.4
3. Open completely	3.8	3.8	3.8
4. 1 day after stage 3	5.0	5.0	5.0

[1]From observation of 5 flowers.

Table 2. Pollen viability measured by cotton blue staining and average self seed set of selected plants.

Plant	No. of Plump, Stained Pollen[1]				Ave. Self Seed Set
	Counts			Ave.	
1	90	95	94	93.0	3.2
8	84	89	91	88.0	0.3
16	96	91	93	93.3	0.0
27	74	68	83	75.0	2.9
61	98	97	96	97.0	1.1
65	72	88	75	78.3	0.7
75	86	97	91	91.3	0.0
113	73	77	81	77.0	0.5
168	96	79	83	86.0	6.4
187	97	96	94	95.7	15.4
216	89	95	79	87.7	1.2
230	96	89	94	93.0	8.1

[1]Counts of 100 pollen grains from 3 flowers.

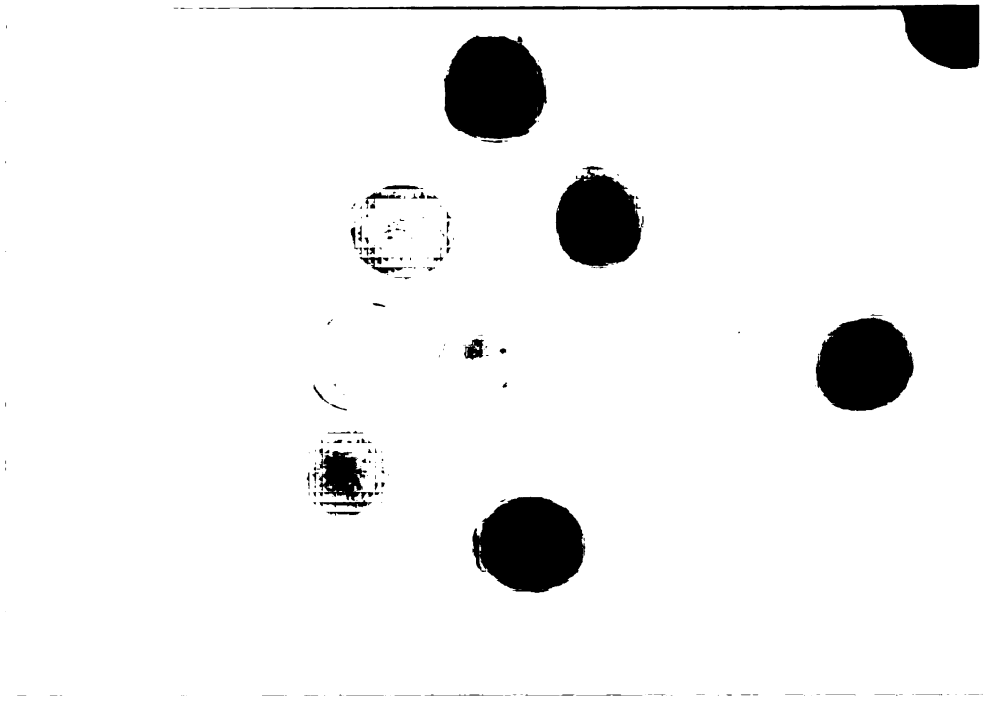


Figure 7. Pollen stained with cotton blue. 400X.

0.5 seed per flower (Table 3). The WI group had the highest average seed set (0.9 seed per flower) and the MI group had the lowest (0 seed per flower). There was no significant difference ($p = 0.1$) in seed set between the groups.

No seed or capsules were set on flowers from the MI group. The seed sets between flowers ranged from 0 to 4 seed on those of the AR group, from 0 to 9 seed on those of the TN group, and from 0 to 13 for those of the WI group. Seeded capsules formed on 5%, 10% and 20% of the AR, TN and WI flowers respectively. Seedless capsules formed on 3%, 20% and 1% of the AR, TN and WI flowers, repectively.

Experiment 3b. No seed were produced on any of the emasculated flowers from either group. Seedless capsules formed on 8% the AR flowers, and no capsules formed on the WI flowers (Table 4).

Experiment 3c. For self-pollinations made on one of the five days beginning at anthesis, there was no significant difference ($p = 0.1$) in seed set between days for either the AR or WI groups. Over the five days, the seed sets between flowers ranged from 0 to 12 seed on those of the AR group and from 0 to 37 seed on those of WI group. Seeded capsules formed on 15% of the AR flowers and on 55% of the WI flowers. Seedless capsules formed on 6 % of the AR flowers and on 3% of the WI flowers.

For each day, there was a significant difference ($p = 0.01$) in seed set between groups (Table 5). For all days

Table 3. Seed set without artificial pollination on flowers from plants[1] studied.

Group	No. of Flowers	Total Seed	Total Seed Capsules	No. of Seed Capsules with No Seed	Ave. Seed per Flwr.
MI	20	0	0	0	0.0
AR	100	12	8	3	0.1
TN	20	12	6	4	0.6
WI	100	89	21	1	0.9
All	240	113	35	8	0.5

[1]Various plants from Michigan (MI), Arkansas (AR), Tennessee (TN), and Wisconsin (WI) groups.

Table 4. Seed set without artificial pollination on emasculated flowers of Arkansas (AR) and Wisconsin (WI) plants.

Group	No. of Flowers	Total Seed	Total seed Capsules	No. of Capsules with No Seed
AR	50	0	4	4
WI	50	0	0	0

Table 5. Effect of flower age at pollination on self seed set for Arkansas (AR) and Wisconsin (WI) plants.

Group	Flower Age at Pollination[1]	No. of Observations	Total Seed	Ave. Seed per Flower
AR	1	40	23	0.6
	2	40	30	0.8
	3	40	30	0.8
	4	40	7	0.2
	5	40	9	0.2
WI	1	40	251	6.3
	2	40	252	6.3
	3	40	215	5.4
	4	40	253	6.3
	5	40	188	4.7

[1]In days; 1=day of anthesis.

combined, the average seed set was 0.5 seed per pollination for the AR group and 5.8 seed per pollination for the WI group.

Experiment 3d(i). For flowers self-pollinated on the day of anthesis at 10 am and 3 pm combined, there was no significant difference ($p = 0.1$) in seed set between flowers which had wet stigmas when pollinated compared to those which had dry stigmas when pollinated, for either the AR or WI groups. Combined over time of pollination, only 7% of the AR flowers and 8% of the WI flowers had wet stigmas on the day of anthesis (Table 6).

Over time of pollination, the average seed set of the AR group was 1.4 seed per pollination for wet stigmas and 0.8 seed per pollination for dry stigmas; the seed sets between flowers ranged from 0 to 11 seed on those which had wet stigmas and from 0 to 23 seed on those which had dry stigmas. Over time of pollination, the average seed set of the WI group was 4.1 seed per pollination for wet stigmas and 5.0 seed per pollination for dry stigmas; the seed sets between flowers ranged from 0 to 16 seed on those which had wet stigmas and from 0 to 39 seed on those which had dry stigmas.

For all flowers which had wet stigmas, seeded capsules formed on 15% from the AR group and on 13% from the WI group; seedless capsules formed on 42% from the AR group and on 22% from the WI group. For all flowers which had dry stigmas, seeded capsules formed on 19 % from the AR group

Table 6. Effect of wet[1] vs dry[2] stigmas on self seed set of flowers on Arkansas (AR) and Wisconsin (WI) plants.

Group	Time of Pollination[3]	No. of Stigmas Wet	No. of Stigmas Dry	Total Seed Wet	Total Seed Dry	Ave. Seed per Flower Wet	Ave. Seed per Flower Dry
AR	10 a.m.	2	48	0	55	0.0	1.2
	3 p.m.	5	45	11	19	2.2	0.4
WI	10 a.m.	2	48	1	284	0.5	5.9
	3 p.m.	6	44	32	175	5.3	4.0

[1]Stigmatic fluid visible on stigma.
[2]No stigmatic fluid visible on stigma.
[3]On day of anthesis.

Table 7. Self seed set on flowers pollinated when their stigmas became wet[1].

Group	Flower Age[2]	Time of Pollination	No. of Wet Stigmas	Total Seed	Ave. Seed per Poln.
AR	1	10 a.m.	3	5	1.7
		3 p.m.	7	0	0.0
	2	10 a.m.	5	17	2.6
		3 p.m.	13	5	0.9
	3	10 a.m.	4	0	0.0
		3 p.m.	7	1	0.1
	4	10 a.m.	5	9	1.8
		3 p.m.	3	0	0.0
	5	10 a.m.	0	-	---
		3 p.m.	1	0	0.0
	Remained dry		2	0	0.0
			50		
WI	1	10 a.m.	0	-	---
		3 p.m.	0	-	---
	2	10 a.m.	6	20	3.3
		3 p.m.	7	24	3.4
	3	10 a.m.	7	28	4.0
		3 p.m.	7	35	5.0
	4	10 a.m.	2	16	8.0
		3 p.m.	6	8	1.3
	5	10 a.m.	0	-	---
		3 p.m.	1	0	0.0
	Remained dry		14	0	0.0
			50		

[1]Stigmatic fluid visible on stigma.
[2]At pollination, in days from anthesis; 1=day of anthesis.

and on 45% from the WI group; seedless capsules formed on 10% from the AR group and on 5% from the WI group.

Experiment 3d(ii). For all days and times combined, pollination on only wet stigmas did not result in significantly different ($p = 0.1$) seed set as compared to that resulting from the pollination on stigmas whether wet or dry (from the results of Experiment 3c; Table 5). Ninety-six percent of the AR flowers produced wet stigmas, 76% occurring after the day of anthesis. Seventy-two percent of the WI flowers produced wet stigmas, all of which occurred after the day of anthesis (Table 7).

The overall average seed set for flowers with wet stigmas was 0.8 seed per pollination for the AR group and 3.6 seed per pollination for the WI group; the seed sets between flowers ranged from 0 to 13 seed on those of the AR group and from 0 to 17 seed on those of the WI group.

For all flowers which had wet stigmas, seeded capsules formed on 17% from the AR group and on 58% from the WI group; seedless capsules formed on 15% from the AR group and on 11% from the WI group. For day of pollination combined over time of pollination, there was no significant difference ($p = 0.1$) in seed set between days for either the AR or WI groups.

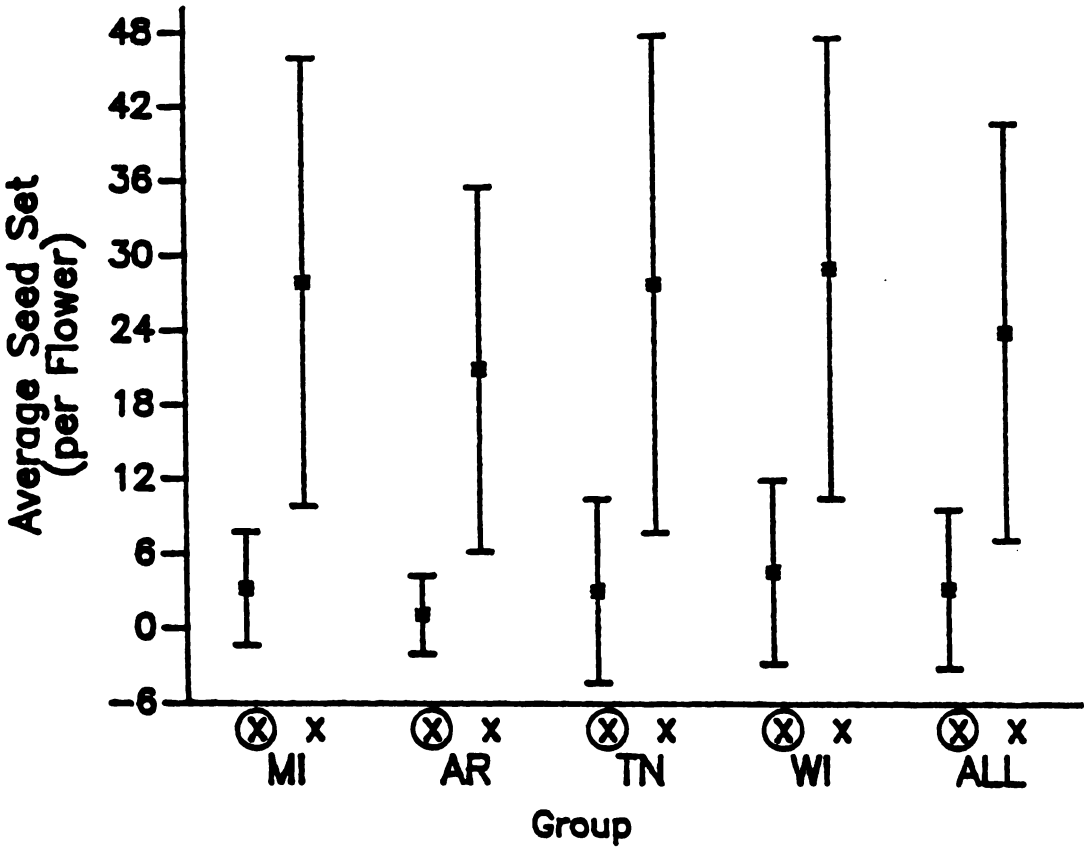
Experiment 3e. The average self seed sets between plants ranged from 0 to 8.3 seed per pollination for those of the AR group, from 0 to 18.7 seed per pollination for those of

the TN group, and from 0 to 21.2 for those of the WI group; the MI plant averaged 3.2 seed per pollination (Appendix 1). The overall average seed set was 3.2 ±6.38 seed per pollination (Figure 8). No seed were set on 32%, 30% and 7% of the plants from the AR, TN and WI groups respectively.

Between flowers, seed sets ranged from 0 to 13 seed on those on the MI plant, from 0 to 23 seed on those of the AR group, from 0 to 34 seed on those of the TN group, and from 0 to 48 seed on those of the WI group; 63% of the pollinations resulted in no seed, and 18% in from 1 to 5 seed (Figure 9). Seeded capsules formed on 40%, 21%, 23% and 49% of the flowers for the MI, AR, TN and WI groups respectively. Seedless capsules formed on 0, 37%, 1% and 16% of the flowers for the MI, AR, TN and WI groups respectively.

There was a significant difference ($p = 0.01$) between the average seed set values of the AR and WI groups. The average seed set values of the TN group were not significantly different ($p = 0.1$) from either the AR or WI groups. For these analyses, the averages were transformed using the equation $(Y + 0.5)^{1/2}$ (Little and Hills, 1978) to reduce the heterogeneity of the variances, caused by the fact that a large proportion of the averages were 0.0 seed per pollination (Appendix 1). The single MI plant was excluded from these analyses.

Experiment 3f. The average cross seed set between plants ranged from 5.4 to 35.4 seed per pollination for those of



Group	Self Averages	Cross Averages
MI	3.2 ±4.57	27.9 ±18.06
AR	1.1 ±3.15	20.8 ±14.69
TN	3.0 ±7.39	27.7 ±20.01
WI	4.5 ±7.38	29.0 ±18.57
ALL	3.2 ±6.38	23.8 ±16.76

[1]Michigan (MI), Arkansas (AR), Tennessee (TN), Wisconsin (WI)

Figure 8. Average seed set of self- and cross-pollinated flowers by group.

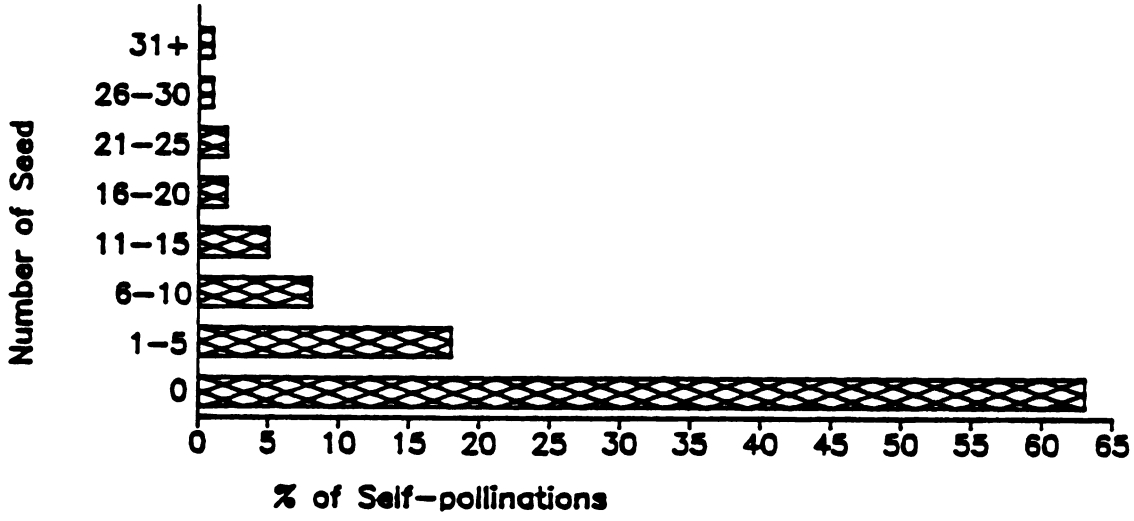


Figure 9. Self seed set distribution.

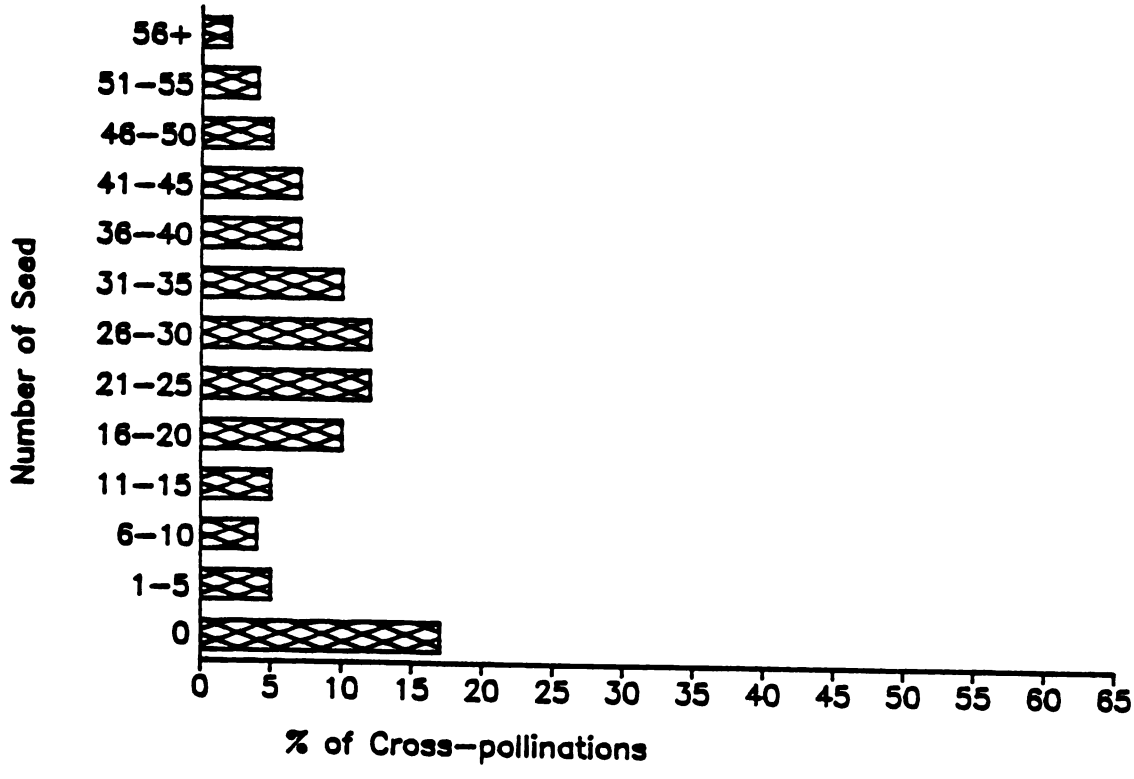


Figure 10. Cross seed set distribution.

the AR group, from 9.7 to 47.7 seed per pollination for those of the TN group, and from 7.0 to 53.6 seed per pollination for those of the WI group; the MI plant averaged 27.9 seed per pollination (Appendix 2). The overall average seed set was 23.8 ±16.76 seed per pollination (Figure 8). Seed were set on all plants from all groups.

For pollinations on flowers from all plants from all groups combined, the individual seed set values ranged from 0 to 73 seeds; 17% of the pollinations resulted in no seed, and 51% in 15 to 40 seed (Figure 10). For the MI, AR, TN and WI groups, seeded capsules formed on 80, 81, 88 and 88% of the flowers, respectively; seedless capsules formed on 0, 10, 0 and 1% of the flowers, respectively.

There was a significant difference ($p = 0.01$) in the average seed set values between the AR and WI groups. The average seed set values of the TN group were not significantly different ($p=0.1$) from either the AR or WI groups. For these analyses, the averages were transformed as described for Experiment 3e, above. The single MI plant was excluded from these analyses.

For the AR, TN and WI groups, each group's self seed set averages were lower than, and significantly different ($p = 0.01$) from, their respective cross seed set averages. This analysis compared the transformed average seed sets for self and cross seed from Experiments 3e and 3f, respectively. The single MI plant was excluded from these analyses.

the AR group, from 9.7 to 47.7 seed per pollination for those of the TN group, and from 7.0 to 53.6 seed per pollination for those of the WI group; the MI plant averaged 27.9 seed per pollination (Appendix 2). The overall average seed set was 23.8 ±16.76 seed per pollination (Figure 8). Seed were set on all plants from all groups.

For pollinations on flowers from all plants from all groups combined, the individual seed set values ranged from 0 to 73 seeds; 17% of the pollinations resulted in no seed, and 51% in 15 to 40 seed (Figure 10). For the MI, AR, TN and WI groups, seeded capsules formed on 80, 81, 88 and 88% of the flowers, respectively; seedless capsules formed on 0, 10, 0 and 1% of the flowers, respectively.

There was a significant difference ($p = 0.01$) in the average seed set values between the AR and WI groups. The average seed set values of the TN group were not significantly different ($p=0.1$) from either the AR or WI groups. For these analyses, the averages were transformed as described for Experiment 3e, above. The single MI plant was excluded from these analyses.

For the AR, TN and WI groups, each group's self seed set averages were lower than, and significantly different ($p = 0.01$) from, their respective cross seed set averages. This analysis compared the transformed average seed sets for self and cross seed from Experiments 3e and 3f, respectively. The single MI plant was excluded from these analyses.

Table 8. Self seed set of F1 plants[1].

Plant	No. of Pollinations	Total Seed	Ave. Seed per Pollination
301	3	0	0.0
302	4	0	0.0
303	2	0	0.0
304	8	0	0.0
305	8	0	0.0
306	6	3	0.5
307	10	2	0.2
308	5	0	0.0
309	10	0	0.0
310	3	5	1.7
All	59	10	0.2

[1]Plants 301 and 308 were the results of crosses between Arkansas and Wisconsin plants. All others were the results of crosses between various Arkansas plants.

Table 9. Pollen and pollen tube characteristics in self-pollinated pistils.

		Hours from Pollination					
		24	48	72	96	120	144
No. of Observ.s		50	73	49	74	59	157
Pollen Qnty.[1,2]	0	0	1	0	0	0	0
	1	2	6	0	0	0	2
	2	98	93	100	100	100	98
Pollen Germ.[1,3]	0	12	12	2	8	3	6
	1	6	8	10	5	2	4
	2	82	80	96	87	87	90
Tube Length[1,4]	0	12	12	2	8	3	6
	1	16	18	10	7	3	2
	2	28	38	43	27	32	29
	3	38	18	6	5	9	11
	4	6	14	39	53	53	52
Ave. Probable Fertilizations[5]		0.1	0.3	2.8	4.4	3.4	1.7

[1]As percent of pistils observed at each time period.
[2]Categories are: 0 = no pollen; 1 = sufficient pollen for 100% fertilization; 2 = very large quantity of pollen.
[3]Categories are: 0 = no pollen germination; 1 = sufficient germination for 100% fertilization; 2 = germination of very large quantity of pollen.
[4]Categories are: 0 = no pollen tubes, or tubes shorter than diameter of pollen; 1 = tubes not to top of ovary; 2 = tubes at top of ovary; 3 = tubes inside of ovary; 4 = tubes enter ovules.
[5]Average number, per pistil, of ovules in which pollen tubes have penetrated.

Table 10. Pollen and pollen tube characteristics in cross-pollinated pistils.

		Hours from Pollination					
		24	48	72	96	120	144
No. of Observ.s		55	95	59	50	23	17
Pollen Qnty.[1,2]	0	0	0	0	0	0	0
	1	9	2	0	0	4	0
	2	91	98	100	100	96	100
Pollen Germ.[1,3]	0	25	9	5	6	4	0
	1	11	7	0	6	17	0
	2	64	84	95	88	78	100
Tube Length[1,4]	0	25	8	5	6	4	0
	1	31	15	3	2	4	0
	2	4	6	0	0	0	0
	3	0	16	0	2	0	0
	4	40	55	92	90	92	100
Ave. Probable Fertilizations[5]		2.0	4.6	21.1	20.0	24.0	22.8

[1]As percent of pistils observed at each time period.
[2]Categories are: 0 = no pollen; 1 = sufficient pollen for 100% fertilization; 2 = very large quantity of pollen.
[3]Categories are: 0 = no pollen germination; 1 = sufficient germination for 100% fertiliation; 2 = germiantion of very large quantity of pollen.
[4]Categories are: 0 = no pollen tubes, or tubes shorter than diameter of pollen; 1 = tubes not to top of ovary; 2 = tubes at top of ovary; 3 = tubes inside of ovary; 4 = tubes enter ovules.
[5]Average number, per pistil, of ovules in which pollen tubes have penetrated.

to 100 germinated pollen; 80% of the selfed pistils and 77% of the crossed pistils had 100+ pollen in their stigmatic cavities. For the 72 to 144 hour time periods combined, 5% of the selfed pistils and of the crossed pistils had no germinated pollen; 5% of the selfed pistils and of the crossed pistils had from 1 to 100 germinated pollen; 89% of the selfed pistils and 91% of the crossed pistils had 100+ germinated pollen in their stigmatic cavities.

Pollen tube growth. (Tables 9, 10). For each the selfed and crossed pistils at each time period, the percentage of pistils which had none or very short pollen tubes was the same as the percentage which had no germinated pollen (pollen germination; category 0).

For selfed pistils at 24 hours, 16% had tubes within the style (category 1), 28% had tubes at the end of the style/top of the ovary (category 2), 38% had tubes in the ovary (category 3), and 6% had tubes in ovules (category 4). For crossed pistils at 24 hours, 31% were category 1, 4% were category 2, 0 were category 3, and 40% were category 4.

For selfed pistils at 48 and 72 hours, 38% and 43% respectively were category 2; 14% and 39% respectively were category 4. For crossed pistils at 48 hours and 72 hours, 6% and 0 respectively were category 2; 55% and 92% respectively were category 4.

For selfed pistils at 96, 120 and 144 hours combined, 29% were category 2 and 52% were category 4. For crossed pistils at 96, 120 and 144 hours combined, 0 were category 2

and 92% were category 4.

Number of pollen tubes in the ovary. (Table 11). Of the selfed pistils at 120 and 144 hours combined, 37% had no tubes in their ovaries, 52% had 1-10 tubes, 9% had 11-49 tubes, and 2% had 50+ tubes. For crossed pistils at 120 and 144 hours combined, 5% had no tubes in their ovaries, 5% had 1-10 tubes, 5% had 11-49 tubes, and 85% had 50+ tubes.

Probable fertilizations. (Tables 9, 10). At 24 hours, the average number of probable fertilizations was 0.1 and 2.0 probable fertilizations per pistil for the selfed and crossed pistils respectively. At 72 hours, the averages were 2.8 and 21.2 probable fertilizations per pistil for the selfed and crossed pistils respectively. At 96, 120 and 144 hours combined, the averages were 2.7 and 21.5 probable fertilizations per ovary for the selfed and crossed pistils respectively (Tables 9, 10).

Experiment 4a(ii).

Pollen quantities and germination. All selfed, crossed, and back-crossed pistils had 100+ pollen. One of the selfed pistils had from 1 to approximately 100 germinated pollen in its stigmatic cavity; the remaining 16 selfed pistils and all of the crossed and back-crossed pistils had 100+ germinated pollen.

Pollen tube growth. Of the 17 selfed pistils, 2 had tubes in their styles, 12 had tubes at the bottom of the style/top of the ovary, and 3 had tubes which penetrated ovules. The 3 crossed pistils and the 4 back-crossed

Table 11. Number of pollen tubes in the ovaries of self- and cross-pollinated pistils.

	Hours from Pollination	No. of Pistils	No. of Tubes in Ovary 0	1-10	11-49	50+
Selfs[1]	120	59	39	41	17	3
	144	157	36	57	6	1
Crosses[1]	120	23	9	9	9	73
	144	17	0	0	0	100

[1]As percent of pistils observed at each time period.

pistils all had tubes which penetrated ovules.

Number of tubes in the ovary. Of the 17 selfed pistils, 14 had no pollen tubes in their ovaries and 3 had from 1 to 10 tubes. Of the 3 crossed pistils, 2 had from 11 to 49 tubes in their ovaries and 1 had 50+ tubes. All four of the back-crossed pistils had 50+ tubes in their ovaries.

Probable fertilizations. The selfed pistils had an average of 0.5 probable fertilizations per pistil. The crossed and back-crossed pistils had averages of 30.0 and 21.5 probable fertilizations per pistil respectively. and

Experiment 4b. Among the plants from all groups combined, the average ovule counts ranged from 28.7 to 80.0 ovules per ovary (Table 12). For all plants from each the AR, TN and WI groups, the averages were 42.3, 50.3 and 60.1 ovules per ovary respectively. The average for the MI plant was 37.1 ovules per ovary. For all plants from all groups combined, the average was 46.4 $\pm$9.48 ovules per ovary.

For the average ovule counts of the plants from each group, there was a significant difference ($p=0.01$) between the averages of the AR group and those of each the TN and WI groups. There was also a significant difference ($p=0.05$) between the averages of the TN and WI groups.

Table 12. Average ovule counts for plants from groups studied[1].

Plt.	No. of Counts	Ave. Ovules[2]	SD	Plt.	No. of Counts	Ave. Ovules	SD
1	60	37.1	5.39	115	10	50.1	6.28
2	43	50.4	9.22	116	8	50.5	4.75
3	6	50.0	9.36	117	9	54.2	7.69
6	5	37.8	10.43	127	3	36.7	3.22
8	11	42.7	9.06	132	3	45.0	4.36
12	12	41.9	3.87	164	4	49.3	9.98
15	14	36.9	4.05	168	6	46.3	4.12
16	11	46.5	5.87	173	4	49.0	4.62
18	4	46.0	3.56	178	3	41.0	7.94
23	4	41.8	9.57	182	6	44.8	8.49
24	9	43.0	10.49	187	17	50.7	9.75
29	6	36.0	6.16	191	23	48.2	5.11
30	5	39.2	6.38	194	10	56.9	7.85
34	14	41.1	10.50	208	4	55.5	9.88
36	5	30.0	3.54	210	3	57.3	2.52
42	12	54.7	9.23	211	8	53.8	6.41
44	3	36.3	5.51	215	3	59.0	8.72
47	3	29.3	4.93	216	8	52.1	8.87
50	16	42.9	5.41	220	6	59.7	5.65
53	3	36.0	10.58	221	9	46.7	9.72
54	13	30.8	3.00	223	6	50.3	11.91
57	4	37.0	3.92	224	10	56.2	7.08
61	19	33.4	5.15	226	6	56.8	9.58
65	10	47.1	6.94	229	7	58.0	7.62
67	20	51.4	7.84	230	10	61.6	7.06
68	16	46.9	6.49	235	9	51.7	6.78
73	9	34.9	5.78	237	7	40.9	6.09
75	8	46.1	4.76	238	4	48.5	2.52
77	3	28.7	3.51	239	3	35.0	2.65
82	23	40.7	5.83	240	4	48.3	5.62
113	4	52.8	13.15	241	3	65.7	14.22
114	12	80.0	9.05				

[1]Plant 1, Michigan; 2-82, Arkansas; 113-117, Tennessee; 127-241, Wisconsin.
[2]Average number of ovules per ovary.

Discussion

The low (self) seed set of *V. pedata* plants in these experiments was difficult to explain *a priori*. The literature contains references to *V. pedata* grown from seed (7, 13; personal communication, Prairie Nursery, Westfield, WI), and from self seed in particular (21). Many if not most other *Viola* species produce abundant self seed, especially those in which cleistogamy and chasmogamy are operative (e.g. *V. riviniana* Rehb., *V. reichenbachiana* Jord. and *V. hirta* L.) (4, 37). The seed set on cleistogamous flowers (necessarily self seed) is much greater than that on chasmogamous flowers (cross and/or self seed) for species in which both systems are operative.

The *Viola* are considered out-crossing species, particularly those which are strictly chasmogamous. The characteristics of their floral morphology and of their insect pollinators promote cross-pollination and limit self-pollination by excluding self pollen from reaching the stigma. However, when self-pollination does occur, it is as likely as cross-pollination to result in seed set (4).

Excluding Emino's work involving inbred lines of *V. tricolor* Hortensis L. (19), the literature contained no references regarding the possible existence of self-

incompability in the Viola. Therefore, the first experiments undertaken (Experiments 1, 2, and 3a-3e) centered on pollen, pollination and seed set in order to determine the reason(s) for low seed set and to determine how seed set could be increased. Only self-pollinations were made, since there was no a priori reason to believe that cross-pollinations would give significantly different results. Because flowering could not be controlled, the self-pollination of available flowers was more readily made than cross-pollination, without the necessity of having a flower at the same stage of development on each of two different plants at the same time.

Anther dehiscence. Anther dehiscence is indicative of pistil receptivity in the flowers of many species (20). Since, in the flowers of V. pedata plants in these studies, the anthers dehisced over a period of approximately 2-3 days (beginning approx. 1 day before anthesis and ending 1 day after), pollination during this period might prove most effective for seed set.

Pollen viability. Using cotton blue stain, the high .ul off percentages of plump, darkly-stained pollen indicate high percentages of viable pollen for the plants studied. Even if these results overestimate the percentage of viable pollen, it is still unlikely that low pollen viability limited the self seed set, since large amounts of pollen (usually several hundred) were transferred to the stigma upon artifiicial

pollination. In addition, the results of the fluorescence microscopy investigations show that large numbers (100+) of germinated (i.e. viable) pollen were found in the stigmatic cavities of 90% of the selfed pistils. Therefore, it is not likely that (low) pollen viability contributed significantly to the problem of low self seed set.

Seed set. For all groups, seed set was very low on flowers which were not artificially pollinated and left undisturbed. As is true of the Viola in general, the physical arrangement of the anthers and their appendages confines the dehisced pollen unless disturbed. After approx. 6 days, depending on weather conditions, the appendages loosen their grip on the style, allowing the pollen to fall into the groove of the spur petal. Some pollen may then spill forward and effect pollination (4). It is possible that air drafts or thrips effected self- and/or cross pollination, resulting in the observed low seed set. The total lack of seed set on emasculated flowers strongly suggests that agamospermy is not operative in these plants of V. pedata. On these flowers, which were not artificially pollinated and left undisturbed, the seed produced was the result of fertilization. The emasculation technique appeared to be very effective at preventing self-pollination, assuming that seed set would result had self-pollination occurred.

Seedless capsule formation on 4 of the 50 emasculated AR flowers would indicate that pollination had probably occured prior to or during emasculation of these flowers. In

many Viola species, pollination without resultant seed set may stimulate capsule formation (22). It is also possible that what appeared to be seedless capsules were actually double ovaries which, because of their relatively large size, look like small capsules.

That seed set on flowers self-pollinated on one of the five days beginning at anthesis did not vary significantly between days suggests that the receptivity of the flowers did not change during this period. Dehisced pollen confined to the anther cone remains viable for 5-8 days in other investigated Viola species (4, 22). Assuming that pollen remains viable in V. pedata flowers for a similar length of time, it is unlikely that a concurrent severe drop in pollen viability and sharp rise in pistil receptivity was responsible for the observed constant seed set between the five days.

The role of stigmatic fluid in stigmatic receptivity is discussed by Beattie for three Viola species. He found that pistils would exude stigmatic fluid, usually on or after day 3 from anthesis. Beattie postulates "that the primary function of the mucilage (stigmatic fluid) was to increase the receptive area for pollen grains" thus "increasing the efficiency of the pollination mechanism" (4). The V. pedata flowers in these investigations exuded stigmatic fluid with irregularity during the five days beginning at anthesis. A flower's stigma might appear "wet" with exudate on any one of the days, but most frequently on days 2-4. The exudate

would not be detectable on the following day. During artificial pollination, more pollen appeared to adhere to wet stigmas than to "dry" stigmas which had no exudate. However, pollination of wet stigmas did not result in increased seed set as compared to that resulting from pollination of dry stigmas. It is possible that stigmatic fluid was present in the cavity of dry stigmas, but not visible. Because the artificial pollination technique placed pollen inside the cavity, sufficient pollen may have adhered to dry stigmas to result in seed sets comparable to that resulting from pollination of wet stigmas.

Inbreeding depression is defined as a decrease in fitness and vigor, resulting from inbreeding imposed on individuals that are normally outbreeding. It is the consequence of increased homozygosity for deleterious recessive genes and the breakup of balanced polygenic systems. Inbreeding depression often results in decreased self seed set, and may be indistiguishable from post-zygotic self-incompatibility systems (34). Cross- pollination between inbred lines often results in vigorous F1 plants, which usually set much more self seed than their inbred parents. Since the F1 hybrid plants of V. pedata in these studies had very low average self seed set values, or set no seed at all, despite their presumed heterogeneity and hybrid vigor, inbreeding depression seems an unlikely explanation for the low self seed set of the parents.

While Experiments 3a-e were being conducted, several

cross-pollinations had been attempted so as to obtain seed for other, unrelated, inheritance studies for flower color (bicolor and concolor types). These crossed flowers produced very large amounts of seed as compared to the amount being produced on selfed flowers in Experiments 3a-e. These large cross seed sets prompted the inclusion of Experiment 3f in which as many plants as possible were scored for cross-compatability.

The highly significant difference in seed set between self- and cross-pollinations had not been anticipated; the literature contained no well-documented cases of self-incompatablity in the Viola (19, 22). The difference between self and cross seed set suggested that a type of self-incompatibility system might be operative in V. pedata.

Fluorescence microscopy. The large difference between self and cross seed set led to the search for differences in pollen and pollen tube characteristics between self- and cross-pollinated pistils (Experiment 4a). The fluorescence microscopy technique employed proved to be very useful for observing pollen, pollen tube growth and ovules.

The observations of pollen and pollen tube growth had to be made with great care so as to achieve the most accurate measurements. First, the stained pistil was examined whole without a coverslip, the stigmatic cavity examined for pollen, and the style examined for tube growth (a "glow" in the style, but this was often not seen due to the thickness of the stylar wall) (Figure 11). The ovary was

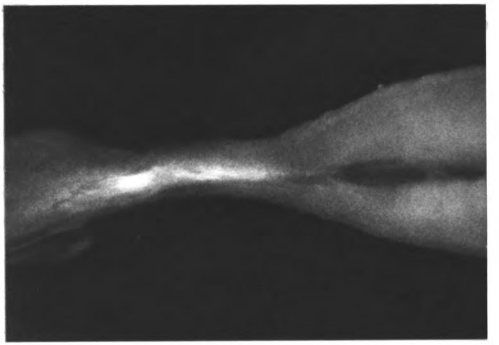


Figure 11. Style (left) and upper ovary (right) of a pistil with glow of fluorescing pollen tubes in style. 20X.

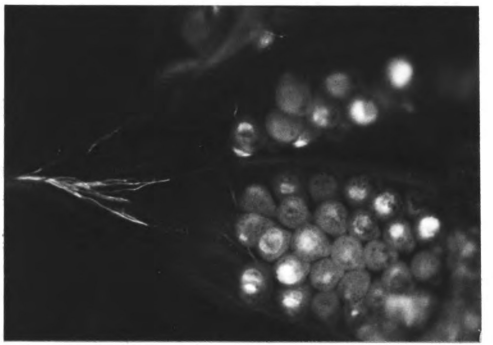


Figure 12. Pistil in Figure 11, split open; most pollen tubes stop at top of ovary. 20X.

then cut open longitudinally, and the sections laid out so that the cut surface faced upward. Figure 12 shows a pistil examined at this stage, without a coverlip. Only a few tubes appear to be entering the ovary. A coverslip placed on the specimen and slight pressure applied. Figure 13 is of the same pistil as Figure 12, which has been slightly flattened, and many more tubes can be seen in the style. Finally, the pistil was flattened even further, revealing many more tubes in the style and several more tubes in the ovary (Figure 14). The flattening process causes the pollen tubes and the ovules to be "squashed" together, making them appear to be in closer proximity than they actually were in the unflattened preparation.

Observations on pollen quantities and germination showed that these were not the limiting factors with regard to the level of seed set. Pollen quantities and germination were high, and nearly the same, for both self- and cross-pollinated pistils. Large numbers of pollen tubes appeared to grow at the same rate, through the hollow style, to the top of the ovary, in both the self- and cross- pollinated pistils. At that point, the similarities ceased.

In the cross-pollinated pistils, large numbers of pollen tubes continued on into the ovary and could be seen entering many of the ovules (Figure 15). In approximately one-third of the self-pollinations, no pollen tubes went beyond the top of the ovary (Figure 16). Although pollen tubes could be found entering the ovaries of the remaing

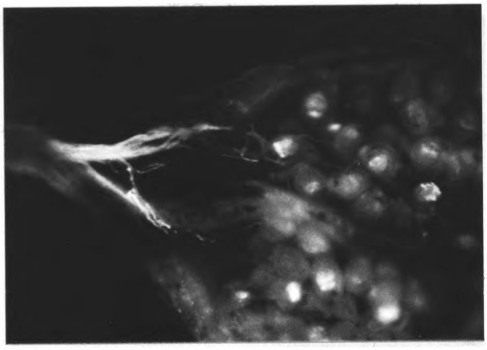


Figure 13. Pistil in Figure 12, slightly squashed; greater number of tubes apparent. 20X.

Figure 14. Pistil in Figure 13, squashed further. 20X.

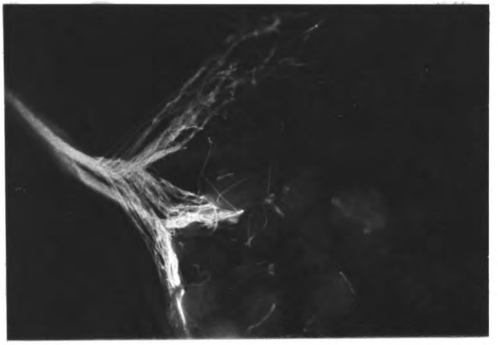


Figure 15. Cross-pollinated pistil showing many pollen tubes in the ovary. 20X

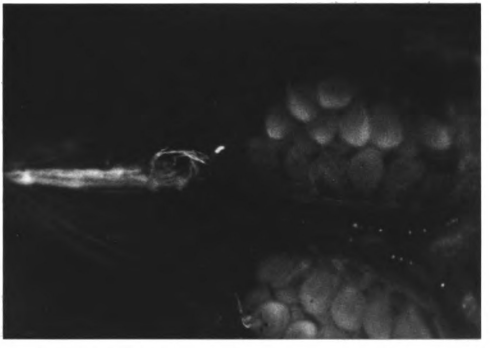


Figure 16. Self-pollinated pistil in which pollen tubes have stopped at top of ovary. 20X.

two-thirds of the self-pollinated pistils, they were far fewer in number than in the cross-pollinated pistils (Figures 17, 18).

The ends of these tubes, and of many of the tubes found in the ovaries of self-pollinated pistils, fluoresced poorly, appeared thin and/or varied in thickness, and often formed large, rounded segments (Figures 19, 20). In comparison, the pollen tubes found in cross-pollinated pistils fluoresced brightly and were of uniform appearance throughout their length.

The pollen tubes appeared to grow along the ovary wall to the placenta between the funiculi and into the ovules (Figures 21, 22, 23). No difference was observed in the appearance of self vs cross pollen tubes as they penetrated ovules.

The observed number of ovules in the ovaries probably underestimates the actual number to some degree due to the loss of some ovules during specimen preparation. From a comparison of the average ovule counts to the self and cross seed set data, it is evident that many ovules do not result in seed, and that self-pollinations made use of far fewer available ovules than did cross-pollinations.

It was evident that a type of barrier was severely restricting the growth of pollen tubes into the ovaries of self-pollinated pistils, or that a barrier was selectively permitting the entry of pollen tubes into the ovaries of cross-pollinated pistils (28, 29, 34). The difference in

Figure 17. Self-pollinated pistil in which a small percentage of pollen tubes have entered ovary. 20X.

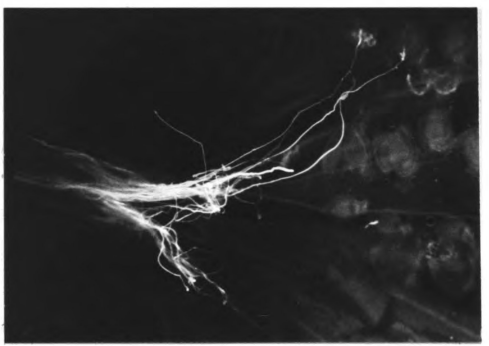


Figure 18. Self-pollinated pistil in which two pollen tubes can be seen entering ovules. 20X.

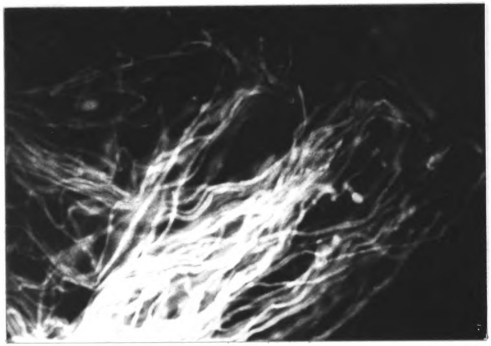


Figure 19. Ends of pollen tubes in a self-pollinated pistil. 100X.

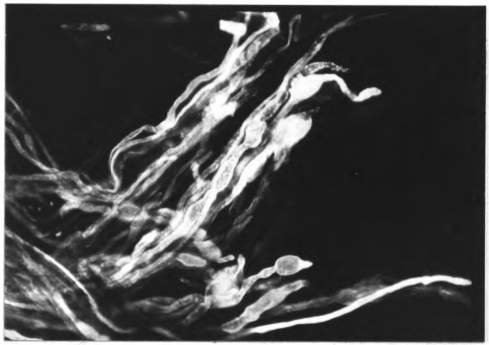


Figure 20. Ends of pollen tubes in a self-pollinated pistil. 100X.

Figure 21. Cross-pollinated pistil with pollen tubes having grown along placenta to ovules. 20X.

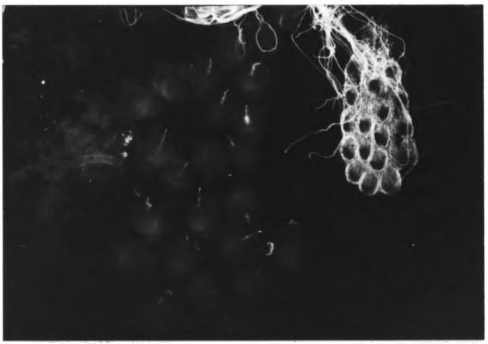


Figure 22. Cross-pollinated pistil with pollen tubes along placenta and in many (detached) ovules. 20X.

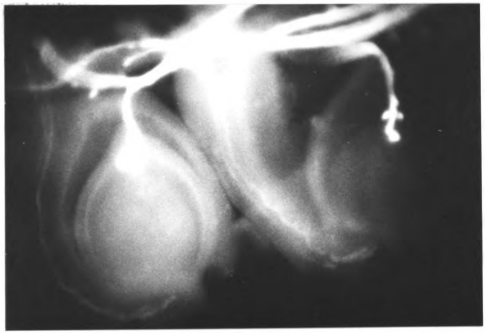


Figure 23. Pollen tubes entering ovules. 100X.

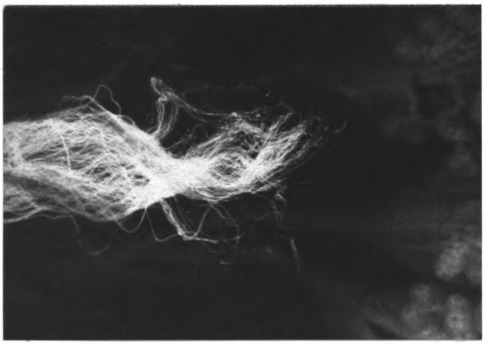


Figure 24. Self-pollinated pistil of an F1 plant with many pollen tubes, all stopping at top of ovary. 20X.

the number of pollen tubes gaining entrance to the ovaries is reflected in the number of pollen tubes entering ovules (probable fertilizations) and in the average seed sets for self- and cross-pollinated pistils. For self-pollinations (averaging 3.19 seed per pistil), an average of 2.41 ovules per pistil had pollen tubes entering them. For cross-pollinations (averaging 23.82 seed per pistil), an average of 21.37 ovules per pistil had pollen tubes entering them.

Pollen tube growth in self-pollinated pistils of the F1 plants also showed a very strong self-incompatability response (Figure 24); this is reflected in their low or nonexistent self seed sets. Crossed and back-crossed pistils of these plants readily allowed pollen tubes to enter the ovaries.

Self-incompatibility in V. pedata. Based on comparisons of self and cross seed set, and on observations of pollen tube growth in selfed and crossed pistils, a genetic self-incompatibility system is proposed to be operative in V. pedata. Inhibition of self pollen tubes growth occurs at the top of the ovary thereby preventing fertilization or greatly reducing its frequency.

The fact that 23% of the cross-pollinations resulted in 0 or 1-5 seed (Figure 10) could be explained if the plants involved were effectively of the same self-incompatibility genotype. It is probable that each the AR, TN and WI groups contained some plants with identical genotypes, due to asexual reproduction. The fact that self-pollinations do

result in some seed set, and that pollen tubes can be found entering some ovaries and ovules of selfed pistils, demonstrates that the self-incompatability barrier is not complete. In fact, the average self seed set values of the plants studied were as high as 21.2 seed, yet 83% of the averages were between 0 and 5.9 seed. Repeated observations of pollen tube growth in self-pollinated pistils of individual plants showed that, on occasion, a number of tubes (most often 1-10, but sometimes more; see Table 11) would gain access to the ovary. The incomplete nature of the proposed self-incompatability system, and how it may be affected by such factors as the (micro-) environmental conditions and plant nutrition, could explain the variable seed set resulting from self- and cross-pollinations on a individual plant. In addition, the fact that self seed yields contain a larger proportion of poorly-filled seed compared to cross seed suggests that a post-fertilization SI system may be operative in V. pedata.

Late-acting self-incompatibility/Ovarian inhibition.

Seavey and Bawa, in a review of late-acting (ovarian) self-incompatiblity in angiosperms, present evidence for such systems in many species (34). They propose that late-acting self-incompatibility systems are not as rare as is currently believed:

> Brewbaker, in pointing out the occurence of hollow styles in many species with self-incompatibility (SI) mechanisms in the ovary, suggested that a lack of intimate contact in such such styles might allow incompatible pollen tube

growth to proceed further than is usual in gametophytic SI systems. Inhibition of pollen tube growth in the ovary would indicate that such inibition may at times operate by chemical mechanisms similar, if not identical, to those that operate in the style of most gametophytic SI systems (9).

Such a late-acting pollen tube inhibition appears to account for the observations of Brock in three species of Lilium. Self pollen tubes were seen to reach the base of the style in all three, and in two species, L. candidum and L. szovitsianum, the ovary enlarged following self-pollination. However, no evidence of endosperm development was found in 312 ovules of L. candidum examined, and only 11 of 468 ovules of L. szovitsianum possessed developing endosperm. Ovaries of the third species, L. pardalinum, did not enlarge following self-pollination (12). Thus, although Brock speculated that some form of post-fertilization inhibition might be operating, as had previously been found in Gasteria (33), it appears more likely that incompatibile pollen tubes were routinely rejected before reaching the ovule. The rare occurence of developing ovules in L. candidum may be an indication that the rejection response is not absolute (12).

Stout and Chandler found similar ovarian responses in Hemerocallis thunbergii and H. citrina, although it is possibile that self pollen tubes interacted with ovules (35). Brewbaker and Gorrez found that the delayed arrest of self pollen in H. thunbergii occurred before the micropyle was reached (10).

In Medicago sativa self pollen tubes do not penetrate into the ovary as far as cross pollen tubes, therefore reaching fewer ovules, and are also less likely than cross pollen tubes to enter the micropyle of an ovule that has been reached (11, 15, 32). Because self-fertilization does occcur, though much less frequently than cross-fertilization, Medicago sativa is considered to be partially (15) or weakly (14) self-incompatible. The fact that in vitro studies of pollen tube growth correlate with in vivo cross-pollination (2), but do not correlate with in vivo observation for self-pollination (36), supports the conclusion that the maternal ovarian tissues are retarding the rate of growth of the self pollen tubes and/or restricting their access to the ovules. (34).

The observations of pollen tube growth in flowers of V. pedata in these investigations suggest a SI system similar

to those found in the Lilium, Hemerocallis, and Medicago species, except that self pollen tubes were inhibited at the top of the ovary in V. pedata. This is the point at which pollen tubes appear to come into close contact with the maternal tissue, thus allowing inhibition by a chemical mechanism similar to those found in gametophytic SI species. The high percentage of poorly-filled self seed, like that found in Medicago sativa (34), suggests the existence of a similar post-fertilization SI system in V. pedata.

It is suggested that further research into this proposed self-incompatability system for V. pedata [e.g. genetics, (micro-) environmental effects] is needed before a breeding program can be established for the development of V. pedata cultivars. Investigations into the population genetics of V. pedata, and into the evolution of the proposed self-incompatability system, would prove interesting and informative, as would the search for self-incompatability in other Viola species. The transfer of self-incompatability to other Viola species may be possible, either through interspecific hybridization or genetic engineering, and prove useful in breeding and seed production efforts.

Summary

The results of these investigations indicate that a self-incompatibility system is operative in Viola pedata, that this is responsible for the overall low (self) seed set of the plants studied, and that seed set can be greatly increased through cross-pollination.

The site of the self-incompatability reaction was found to be in the area where the hollow style gives way to the ovary cavity. Either the pollen tubes in self-pollinated pistils are greatly restricted from entering the ovary, or tubes of cross-pollinated pistils are selectively permitted to enter. Since some self seed was obtained, the self-incompatability reaction is not complete. Self-compatible and/or semi-compatible genotypes may exist.

Pollen quantities and viability, the timing of pollination, and the presence/abscence of visible stigmatic fluid do not appear to be critical factors limiting (self) seed set. It was found that agamospermy is highly unlikely to be operative in this species.

Further research into the genetics and expression of the proposed self-incompatability system is suggested. In addition, investigation into seed germination requirements for this species is highly recommended.

It is suggested that Viola pedata would make a welcome addition to the garden, and possibly to the pot-plant market, if suitable seed-produced cultivars can be developed.

APPENDICES

Appendix 1. Average seed set[1] of self-pollinated flowers by plant.

Plt[2]	Seed Set	Plant	Seed Set	Plant	Seed Set	Plant	Seed Set
1	3.2	59	0.0	139	1.0	200	2.5
2	0.9	60	0.7	140	0.0	201	5.7
3	0.0	61	1.1	141	0.0	202	11.9
4	0.6	65	0.7	143	0.0	203	3.7
5	3.2	67	0.0	148	0.0	204	2.4
6	0.0	68	0.8	149	2.1	205	7.9
8	0.3	71	0.3	151	0.0	206	0.7
10	0.2	72	6.2	156	6.4	208	2.5
11	1.6	73	1.2	157	2.9	209	21.2
14	0.0	74	5.0	159	2.9	210	10.1
15	0.0	75	0.0	160	2.0	211	18.2
16	0.0	76	5.0	161	4.5	213	1.3
18	0.0	77	1.2	162	0.5	214	0.0
19	2.5	79	1.7	163	4.0	215	14.0
20	0.0	80	0.0	164	4.2	216	1.2
21	0.1	81	1.2	165	2.9	218	1.3
23	0.7	82	1.3	166	3.8	219	15.3
24	8.3	85	0.8	167	2.6	220	0.2
25	0.1	86	0.1	168	6.4	221	0.6
27	2.9	113	0.5	172	1.7	222	3.7
28	1.0	114	0.2	173	2.5	223	0.2
29	1.5	115	0.0	174	2.8	224	4.1
30	4.5	116	3.3	175	3.2	225	1.2
31	0.9	117	0.1	178	5.5	226	0.2
32	0.0	118	0.0	180	6.9	227	0.8
33	1.6	119	6.6	181	1.6	228	4.7
34	4.9	120	0.7	182	6.3	229	13.7
35	0.0	121	0.0	184	1.7	230	8.1
36	0.0	122	18.7	185	0.5	231	5.2
37	0.9	123	0.7	186	4.3	232	8.0
39	1.3	124	1.3	187	15.4	233	0.8
40	0.2	125	2.7	188	10.4	234	1.2
41	0.0	126	2.4	189	9.4	235	1.0
42	0.3	127	1.4	190	1.1	236	17.4
44	1.4	129	1.1	191	10.9	237	6.8
47	0.0	130	0.0	192	5.6	238	3.3
48	0.0	131	0.5	193	2.9	239	13.7
50	0.5	132	4.4	194	18.8	240	9.9
53	0.0	133	1.4	195	2.9	241	16.2
54	1.5	134	0.9	196	1.5	242	2.2
56	0.0	135	2.7	198	1.0		
57	2.5	136	3.7	199	3.2		

[1]Average of 10 self-pollinated flowers for each plant.
[2]Plant 1, Michigan; 2-86, Arkansas; 113-122, Tennessee; 123-242, Wisconsin.

Appendix 2. Average seed set[1] of cross-pollinated flowers by plant.

Plt[2]	No. X's[3]	Total Seed	Ave. Seed Set	Plant	No. X's	Total Seed	Ave. Seed Set
1	30	836	27.9	114	3	32	10.7
2	37	1124	30.4	115	5	132	26.4
3	10	158	10.8	116	6	286	47.7
4	58	882	15.2	117	6	99	16.5
5	9	263	29.2	118	5	123	24.6
8	16	491	30.7	119	7	234	33.4
12	21	473	22.5	121	3	29	9.7
14	7	83	11.9	122	8	310	38.8
15	10	202	20.2	127	4	84	21.0
16	23	500	21.7	130	3	56	18.7
23	7	103	14.7	178	4	69	24.8
24	3	76	25.3	181	4	154	38.5
25	3	93	31.0	187	28	972	34.7
27	16	384	24.0	188	4	147	36.8
29	6	122	20.3	189	4	35	8.8
30	7	248	35.4	190	4	109	27.3
32	8	140	17.5	191	4	127	31.8
33	3	60	20.0	193	3	71	23.7
34	35	916	26.2	194	13	464	35.7
35	3	40	13.3	205	4	50	12.5
36	7	38	5.4	206	3	131	43.7
37	9	173	19.2	211	3	136	45.3
39	4	62	15.5	215	9	289	32.1
41	10	128	12.8	216	5	140	28.0
42	6	92	15.3	218	3	78	26.0
44	19	309	16.3	220	3	66	22.0
50	9	88	9.8	223	4	150	37.5
54	41	699	17.1	224	6	292	48.7
56	10	243	24.3	225	3	91	30.3
61	14	299	21.4	226	3	52	17.3
67	16	289	18.1	229	9	226	25.1
68	6	146	24.3	230	4	165	41.3
71	8	98	12.3	231	4	116	29.0
72	5	60	12.0	232	4	150	37.5
73	5	150	30.0	235	7	220	31.4
75	15	381	25.4	236	10	390	39.0
76	4	107	26.8	237	7	134	19.1
80	20	572	28.6	239	3	21	7.0
82	5	80	16.0	240	4	146	36.5
85	5	129	25.8	241	7	375	53.6
113	5	105	21.0	242	10	200	20.0

[1]Average of at least three crosses with pollen from at least three different male parents.
[2]Plant 1, Michigan; 2-85, Arkansas; 113-122, Tennessee; 123-242, Wisconsin.
[3]Number of cross-pollinations made.

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