PARTIAL SELF-INCOMPATIBILITY AND THE COLLAPSE OF FERTILE OVULES AS FACTORS AFFECTING SEED FORMATION IN ALFALFA [1]

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INTRODUCTION

Various investigators have confirmed the fact, since Piper et al. (*10*) [3] first reported it, that alfalfa (*Medicago sativa* L.) forms seed more freely after cross-pollination than after self-pollination. The authors (*4*) in a preliminary account, have shown that two probably distinct phenomena are involved. In the first place, it is found that alfalfa is partly self-incompatible. That is to say, male gametophytes are less effective in accomplishing fertilization in the plant from which they arise than in unrelated individuals. Moreover, there is a difference in survival of ovules containing inbred and hybrid embryos and endosperms, the former class being much more prone to collapse during development than the latter. The observations upon which these findings rest are presented in detail in the present paper and are considered in relation to the problem of seed setting in alfalfa.

Self-incompatibility is usually measured in terms of fruit and seed production. This may not be adequate in alfalfa in view of the possibility that ovule abortion, which also has a major effect on fruit and seed production, may be unrelated to self-incompatibility and may vary independently of it. The two phenomena have been separated in the present study by a histological study of the pistil following the two types of matings.

MATERIALS AND METHODS

Seven plants derived by self-pollination from six unrelated individuals selected from commercial varieties of alfalfa were used in the experiment. They were brought into flower in pots in a greenhouse at 70° to 80° F. in April, supplementary illumination being used to give a photoperiod similar to that in midsummer. The matings were made at the height of the flowering period. Newly opened flowers were castrated by clipping off the standard, forcing the sexual column out of the keel, and removing the pollen by suction. Some of the flowers were then selfed, using pollen from other flowers on the same respective plants. The remaining castrated flowers were cross-pollinated, the pollen being taken in each case from a different and unrelated plant within the group. The matings were so arranged that each of the plants, in addition to being selfed, served as a pistillate and as a staminate parent in the crosses. Intimate contact between the stig-

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[3] Italic numbers in parentheses refer to Literature Cited, p. 472.

matic secretion and the pollen was assured by applying the pollen with toothpicks tipped with a strip of fine emergy cloth.

The pistils were collected at 30, 48, 72, 96, 120, and 144 hours after pollination, fixed in Karpechenko's modification of Navashin's fluid, and embedded in paraffin. After the material had been sectioned and stained with Delafield's haematoxylin, the following data were taken on each pistil, regard being given to the serial order of the ovules from apex to the base of the ovary: Position of the most advanced pollen tubes, fertility of the ovules, stage of development of the proembryo or embryo, and fertile ovules collapsing. The technique employed did not permit of following pollen-tube growth in the style. Within the cavity of the ovary, however, the pollen tubes grow along the surface of the ventral suture, and, at this stage, the tips of the longest tubes can be distinguished clearly, although the distribution of the less advanced tubes cannot be determined accurately. About nine pistils per plant were taken at each collection in the self-pollinated series and about six pistils per plant in the crossed group.

SEED PRODUCTION FOLLOWING SELF- AND CROSS-POLLINATION

The comparative fertility on self- and cross-pollination of the seven plants used, measured in terms of mature seeds produced, is shown in table 1. The data are based on a separate series of matings made during the same period as the test pollinations. The flowers in the selfed series, however, were not castrated, and the pollen used in the crosses was derived mostly from unrelated plants not otherwise included in these experiments. It is not believed that either of these circumstances should influence the results significantly.

TABLE 1.—*Fertility measured in terms of mature seeds produced, following self- and cross-pollination*

Plant (pistillate parent)	Self-pollination				Cross-pollination			
	Flowers	Pods	Seeds	Seeds per flower	Flowers	Pods	Seeds	Seeds per flower
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
A [1]	107	66	164	1.53				
B	74	26	43	.58	30	27	81	2.70
C	51	9	8	.16	24	22	133	5.54
D	105	45	78	.74	54	45	216	4.00
E	67	49	118	1.76	24	19	120	5.00
F	35	15	33	.94	76	69	450	5.92
G	43	15	27	.63	80	59	310	3.87
Total or average	375	159	307	.80	288	241	1,310	4.50

[1] Omitted from total.

Omitting plant A, for which the data are incomplete, it may be seen from table 1 that, on an average, about five and one-half times as many mature seeds developed per flower pollinated after crossing as after selfing. The values vary rather widely from plant to plant, particularly in the selfed series, but in the case of each individual a large increase is shown on crossing. This result is in conformity with the findings of Piper et al. (*10*), Frandsen (*8*), and Carlson (*5*).

The fertility differential between the crossed and selfed series is much wider when measured in terms of seeds formed per flower pollinated than in terms of pods set per flower pollinated. This follows from the fact that the pods formed after selfing contained only 1.93 seeds each, on an average, whereas those in the cross-pollinated series contained 5.44 seeds.

OVULE FERTILITY

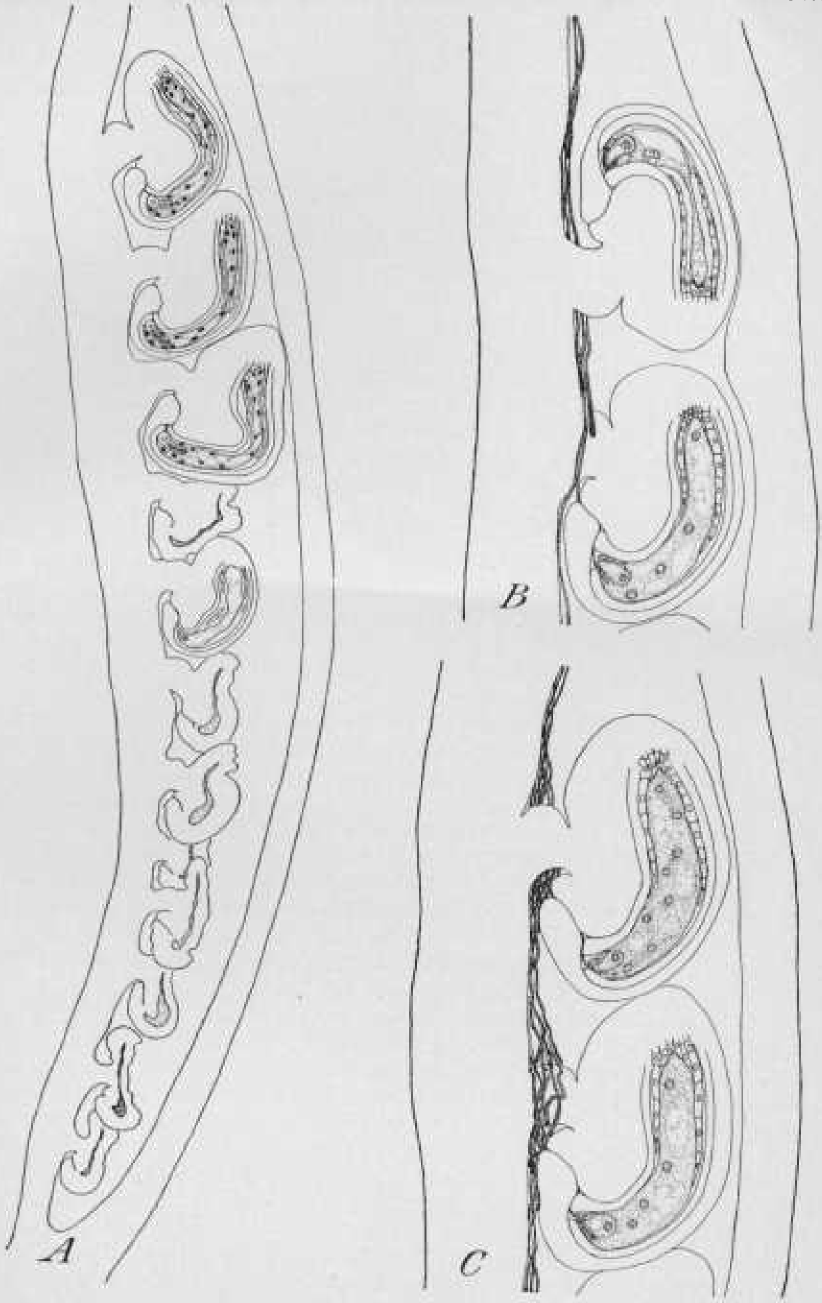
A primary cause of the disparity in the number of seeds that develop after self- and cross-pollination is found in the difference in the proportion of ovules that become fertile following the two types of matings. The ovary of alfalfa usually contains 10 to 12 ovules arranged alternately along the ventral suture in serial order. The ovules are comparatively uniform in size and, almost without exception, develop normal appearing embryo sacs. A necessary condition for the development of the seed is that fertilization of the egg within the ovule occur. A histological study of the ovary during the period immediately following completion of pollen-tube growth enables one to measure directly the frequency with which fertilization takes place. Failure of fertilization can thus be separated from other factors subsequently affecting fertility.

Just prior to fertilization the mature ovule is in a quiescent state. There is little, if any, evidence of nuclear or cell division in any of its parts. The pollen tube enters the embryo sac between the apices of the synergids and the egg. In the process of fertilization one male gamete nucleus fuses with the egg nucleus and the other unites with the fusing polar nuclei. Coincindental with fertilization active nuclear and cell division is stimulated in the integuments and funiculus and there is a considerable increase in the size of the ovule within a short period of time. The primary endosperm nucleus has oftentimes divided twice and there are four endosperm nuclei present at the time of the division of the zygote.

The fertilized ovules at 48 hours after pollination may be distinguished from those that have not been fertilized by the increase in size of the ovule, the development of the multinucleate endosperm, and the presence of a proembryo. If fertilization does not occur there is no further development of the ovule, the embryo sac collapses and ultimately the entire ovule, so that only a vestige of it remains at the later stages of the developing pod (pl. 1, *A*).

At 72 hours after pollination pollen tubes, which may be seen at certain earlier stages, can no longer be found within the cavity of the ovary. Fertilization, therefore, has ended, and a legitimate estimate of the frequency with which the process occurs after a given mating may be made. The results of an examination of ovaries collected at 72, 96, 120, and 144 hours after pollination are presented in table 2 and depicted graphically in figure 1. The samples collected at the different times provide the basis for four independent estimates of the percentage of fertile ovules in each plant in each of the two series.

It is evident from table 2 that a much larger proportion of the ovules become fertile after cross-pollination than after self-pollination. The respective mean values are 66.2 and 14.6 percent. Furthermore, the values for the two series are separated by a wide gap. The most fertile plant in the selfed series, B, shows 21.5 percent of the ovules containing a fertilized egg. The least fertile plant on crossing, which likewise proves to be B, has over twice as many fertile ovules. A statistical examination of the data brings to light additional relations not otherwise clearly apparent.



A, Median longitudinal section of an ovary 72 hours after self-pollination. The three apical ovules and the fifth ovule have been fertilized, the latter being in a stage of collapse. The remaining ovules have not been fertilized and are in stages of degeneration. × 425. *B*, Median longitudinal section of a portion of an ovary showing the two apical ovules 30 hours after self-pollination. The second ovule has been fertilized. The pollen tubes are few in number. × 850. *C*, Median longitudinal section of a portion of an ovary showing the two apical ovules 30 hours after cross-pollination. Both ovules have been fertilized. Pollen tubes are abundant, especially in the regions of the micropyles. × 850.

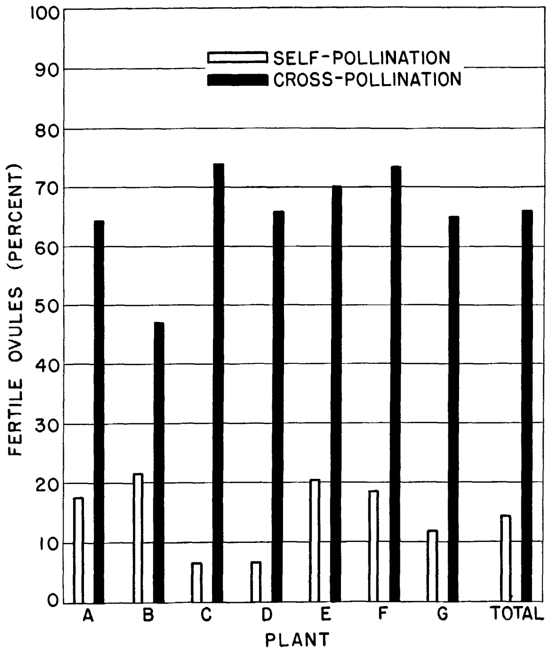


FIGURE 1.—Frequency of fertilization in individual plants following self-pollination and cross-pollination. Data based on pistils collected at 72, 96, 120, and 144 hours after pollination.

TABLE 2.—*Comparative fertility of ovules in individual plants following self- and cross-pollinations; data based on collections at 72, 96, 120, and 144 hours after pollination*

Plants selfed or crossed	72 hours			96 hours			120 hours			144 hours			Total		
	Total	Fertile		Total	Fertile		Total	Fertile		Total	Fertile		Total	Fertile	
	No.	*No.*	*Pct.*	*No.*	*No.*	*Pct.*	*No.*	*No.*	*Pct.*	*No.*	*No.*	*Pct.*	*No.*	*No.*	*Pct.*
Selfed:															
A	58	10	17.2	82	21	25.6	58	4	6.9	12	2	16.7	210	37	17.6
B	44	14	31.8	58	10	17.2	26	5	19.2	44	8	18.2	172	37	21.5
C	76	10	13.2	79	4	5.1	82	5	6.1	60	1	1.7	297	20	6.7
D	82	5	6.1	73	2	2.7	72	6	8.3	20	4	20.0	247	17	6.9
E	65	9	13.8	43	15	34.9	47	6	12.8	37	9	24.3	192	39	20.3
F	136	29	21.3	167	28	16.8	153	25	16.3	130	27	20.8	586	109	18.6
G	127	10	7.9	137	12	8.8	125	23	18.4	54	10	18.5	443	55	12.4
Total	588	87	----	639	92	----	563	74	----	357	61	----	2,147	314	14.6
Crossed:															
A×B	58	34	58.6	81	53	65.4	77	52	67.5	73	48	65.8	289	187	64.7
B×C	58	25	43.1	61	26	42.6	60	30	50.0	54	29	53.7	233	110	47.2
C×D	56	38	67.9	77	68	88.3	48	28	58.3	48	37	77.1	229	171	74.7
D×E	71	43	60.6	52	32	61.5	53	38	71.7	82	58	70.7	258	171	66.3
E×A	50	34	68.0	44	32	72.7	54	37	68.5	57	41	71.9	205	144	70.2
F×G	92	67	72.8	81	54	66.7	50	40	80.0	86	67	77.9	309	228	73.8
G×F	90	78	86.7	92	55	59.8	72	38	52.8	50	27	54.0	304	198	65.1
Total	475	319	----	488	320	----	414	263	----	450	307	----	1,827	1,209	66.2

A reduction of the present data in original form by the analysis of variance technique might prove somewhat misleading in view of the fact that percentages have different variances depending on their values and the number of observations on which they are based. A more exact analysis may be made by first transforming the variates to θ values, according to the method proposed by Bartlett (*1*) and Bliss (*2*). This transformation has been made, and the analysis of variance procedure then applied. The analysis of variance based on the weighted θ's corresponding to the observed percentages of fertile ovules is presented in table 3.

TABLE 3.—*Analysis of variance based on weighted θ's for fertility of ovules following self- and cross-pollination*

Type of mating	Source of variance	Degrees of freedom	Sum of squares	Mean square	F	$F_{.05}$	$F_{.01}$
Self-pollination	Between plants	6	14.895378	2.4826	4.365	2.57	3.81
	Between replicates within plants.	21	11.942734	.5687			
	Subtotal	27	26.838112				
Cross pollination	Between plants	6	13.805651	2.3009	3.245	2.57	3.81
	Between replicates within plants.	21	14.890777	.7091			
	Subtotal	27	28.696428				

The first question of interest to which an answer may be sought is whether the increase in the proportion of fertile ovules following cross-pollination is actually larger than might arise from random sampling alone. Although the number of flowers used and the percentage of fertile ovules observed in the self- and cross-pollinated series vary rather widely, as may be seen in table 2, it is evident from table 3 that the estimated variances for the two groups, i. e., 0.5687 and 0.7091, are similar. The difference, in fact, is nonsignificant, since $F=1.25$, and for significance it would have to exceed 2.08. Consequently, these estimates may be pooled, giving 0.639 as the estimated variance of a single θ value. This corresponds to an estimated standard deviation of 0.709. The weighted mean θ's for the two groups are as follows:

Self-pollination=0.379, based on 2,147 ovules.
Cross-pollination=0.956, based on 1,827 ovules.

The estimated standard deviation between these means, therefore, is 0.025, from which $t=23.1$. As this value of t far exceeds the 1-percent value (2.7) for 42 degrees of freedom, it may be concluded with considerable confidence that the self- and cross-pollinated groups differ significantly in proportion of fertile ovules.

The further question may be asked whether the frequency of fertile ovules is significantly increased by cross-pollination in the case of each plant. Since standard errors for the percentages are not available the significance of the differences must be tested on the basis of the mean θ values. These values for the several plants in the self- and cross-pollinated series are shown in table 4. The largest standard deviation for two mean values for the same plant, but for different series, is clearly less that $0.799\sqrt{\frac{1}{172}+\frac{1}{205}}$, so that an excessive minimal significant difference is found by multiplying this by 2.704 (the 1-percent value for t, with 42 degrees of freedom). The resulting value is 0.223. Referring to table 4, it is seen that the smallest actual difference is 0.278 for plant B. Since this is significant it follows that the increase in percentage of fertile ovules on cross-pollination for each of the other plants is likewise significant.

TABLE 4.—*Mean θ values and percentage of fertile ovules as estimated by inversion from the θ values for the individual plants in the self- and cross-pollinated series*

Plant	Self-pollination			Cross-pollination		
	Total ovules	Weighted mean θ	Estimated fertile ovules	Total ovules	Weighted mean θ	Estimated fertile ovules
	Number		*Percent*	*Number*		*Percent*
A	210	0.423	16.8	289	0.935	64.7
B	172	.479	21.2	233	.757	47.2
C	297	.251	6.2	229	1.054	75.6
D	247	.254	6.3	258	.952	66.4
E	192	.459	19.6	205	.994	70.3
F	586	.445	18.5	309	1.035	73.9
G	443	.354	12.0	304	.950	66.2

A similar procedure may be followed in testing whether the different plants vary significantly in the proportion of ovules becoming fertile under each of the two systems of mating. It is found that the largest minimal 5-percent difference is 0.164 and the smallest minimal 5-percent difference is 0.100. Applying these minimal significant differences, it may be stated that, at the 5-percent level of significance, plants C and D are significantly less fertile than plants A, B, E, and F, and that the latter do not differ significantly from each other. Likewise, D does not differ significantly from C or from G. Furthermore, G is not significantly different from A and F. The exact minimal significant differences between G, on the one hand, and C, E, and B, on the other, are 0.118, 0.135, and 0.141, respectively. Plant G, therefore, does not differ significantly from any of these plants. The comparative fertility relations in the self-pollinated series may be summarized as shown in table 5.

TABLE 5.—*Comparative fertility relations in self-pollinated plants A to G*[1]

Plant	A	B	C	D	E	F	G
A	0	0	+	+	0	0	0
B	0	0	+	+	0	0	0
C	–	–	0	0	–	–	0
D	–	–	0	0	–	–	0
E	–	–	+	+	0	0	0
F	0	0	+	+	0	0	0
G	0	0	0	0	0	0	0

[1] Plant A is significantly more fertile than C, and the latter is significantly less fertile than F. + denotes significantly greater, – significantly less, and 0 nonsignificance.

Proceeding similarly it may be shown that in the cross-pollinated series, plant B is significantly less fertile than the other individuals, and that these latter do not differ significantly from one another.

FERTILITY OF THE OVULES IN RELATION TO POSITION IN THE OVARY

A striking fact which is revealed by histological study of the pistils of alfalfa following self- and cross-pollination is a declining gradient in fertility of the ovules from the apex to the base of the ovary. The observations bearing on this point are summarized in table 6 and figure 2. In the selfed series, about one-third of the ovules in position 1,

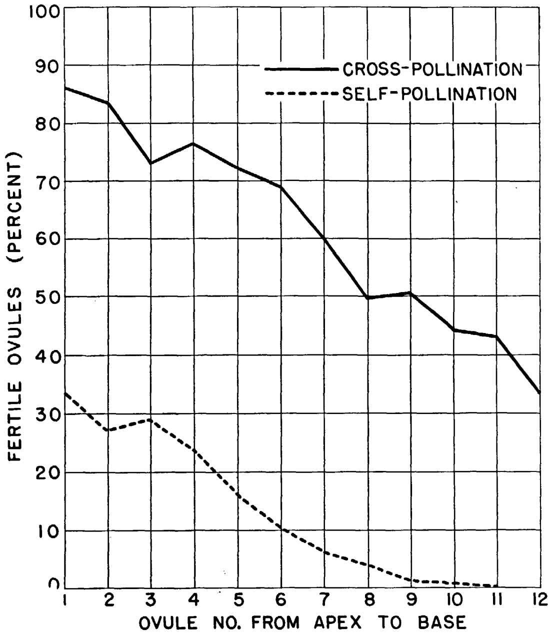


FIGURE 2.—Percentage of fertile ovules according to position in the ovary, following self- and cross-pollination. Averages based on pistils collected at 72, 96, 120, and 144 hours after pollination for seven plants. The apicale ovule is No. 1.

that is, at the apex of the ovary, become fertile. Only about one-half as many in position 5 become fertile, and the frequency declines to zero towards the base.

The fertility gradient follows an almost parallel course in the cross-pollinated series, although the proportion of ovules becoming fertile in each position is much higher than after selfing. In position 1, for example, about 85 percent of the ovules contain fertilized eggs; in position 5 the value has declined to about 72 percent, and at the base of the ovary about one-third of the ovules become fertile.

POLLEN-TUBE GROWTH IN THE OVARIAN CAVITY

The explanation for the difference in the proportion of ovules that become fertile after self- and cross-pollination lies, in part, in the extent of pollen-tube growth following the two types of matings. As mentioned earlier, the position of the ends of only the most advanced pollen tubes within the ovarian cavity can be determined definitely by the technique used. This evidence, nevertheless, is sufficient to show clearly that restricted pollen-tube growth is a factor affecting fertility after self-pollination.

TABLE 6.—*Fertility of the ovules in relation to position in the ovary following self- and cross-pollination*

[Data based on collections at 72, 96, 120, and 144 hours]

Ovary No.[1]	Self-pollination of ovules			Cross-pollination of ovules		
	Total	Fertile		Total	Fertile	
	Number	*Number*	*Percent*	*Number*	*Number*	*Percent*
1	205	70	34.1	181	156	86.2
2	205	56	27.3	181	152	84.0
3	205	59	28.8	181	133	73.5
4	205	49	23.9	181	139	76.8
5	205	33	16.1	181	131	72.4
6	205	21	10.2	181	125	69.1
7	205	13	6.3	181	109	60.2
8	205	9	4.4	181	90	49.7
9	191	3	1.6	159	80	50.3
10	172	1	.6	131	58	44.3
11	124	0	0	65	28	43.1
12	40	0	0	24	8	33.3
Total	2,167	314	14.5	1,827	1,209	66.2

[1] The apical ovule is No. 1.

The frequency distributions of pistils with reference to the farthest point of pollen-tube advance at 30 hours after pollination in the selfed and crossed series are shown in table 7. Position of the pollen-tube tips is recorded in terms of the particular ovule reached, 1 being the apical ovule. Length is expressed accordingly in "ovule units." The entries in the 0 column relate to pistils in which no pollen tubes were found within the ovary. There are two possible explanations for these cases. Either the pollen tubes were somewhat tardier in development and had not yet emerged from the base of the style, or the pistils had been so injured during castration and pollination that normal growth could not occur. It makes little difference in the conclusions to be drawn later whether these cases are included or omitted. Accordingly, they have been omitted from the summary columns.

It will be observed from table 7 that, in the case of each plant, the pollen tubes are farther advanced after crossing than after selfing. The difference, however, is much larger in some cases than in others. In plant D, selfed, for example, the longest pollen tubes have advanced on an average only slightly beyond the second ovule; whereas, in the cross-pollinated pistils from this individual, the longest tubes have reached the tenth ovule. The longest pollen tubes in plant G, on the other hand, are only slightly longer after hybridization than after selfing. There is little doubt, however, that the difference in length of the longest pollen tubes in the two series is statistically significant, as the comparison in the following paragraph shows.

TABLE 7.—*Frequency distribution of pistils with reference to farthest point of pollen-tube advance at 30 and 48 hours after self- and cross-pollination, respectively*

Mating	Pollen tubes penetrating to ovule No.—													Total pistils	Mean
	0 [1]	1	2	3	4	5	6	7	8	9	10	11	12		
30 HOURS															
	No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
A selfed	2		1	2	2	2	1							8	4.0
A × B	1										1	3	1	5	11.0
B selfed		3			1		1	2	2					9	4.8
B × C									3	5				8	8.6
C selfed	1			1	1	3		2						7	5.1
C × D	1			1							1	3	1	6	9.7
D selfed		3	2	2	2									9	2.3
D × E											6			6	10.0
E selfed					1		2	2	1	1				7	6.7
E × A							1		1	2	1			5	8.4
F selfed	2			2	3	3	1							9	4.3
F × G	1				1		1	1	1	1	3			8	8.0
G selfed	5	1	1		3			1	1					7	4.3
G × F	4			2	2	4	4	1						13	5.0
48 HOURS															
A selfed					1	2	2	1	1					7	5.9
A × B									1			1	4	6	11.1
B selfed							2			5				7	8.1
B × C									1	4	2			7	9.1
C selfed				1		1	2	2						6	5.7
C × D												4	2	6	11.3
D selfed		1	1	1	2	1								6	3.2
D × E					1						2	4	1	8	10.0
E selfed									3	2	2			7	8.9
E × A									3	3	1			7	8.7
F selfed			2	1	2	5	5	2	3					20	5.4
F × G								1			4	1		6	9.7
G selfed	4			2	2	4	4	1						13	5.0
G × F	1										4	1		5	10.2

[1] Not included in summary columns; relates to pistils in which no pollen tubes were found within the ovary.

The weighted mean length of the longest pollen tubes of the seven plants in the selfed series is 414 ovule units, and the estimated variance is 13.75 ovule units (6 degrees of freedom). In the crossed series the corresponding values are 8.1 and 34.34. Since $F=2.50$, which is less than the 5-percent value, the two variances can be pooled, giving 24.04 as the estimated variance of a single mean. The standard deviation is, therefore, 4.90, and the standard deviation of the difference between the means of the two series is 0.93. It follows that $t=3.978$. Since this value of t exceeds that for the 1-percent point, 3.055, with 12 degrees of freedom, it may be inferred that at 30 hours after pollination the mean length of the longest pollen tubes in the crossed series is significantly greater than that in the selfed group.

An independent series of observations was made on the pollen tubes in the pistils collected at 48 hours after pollination. The data are presented in table 7. The results, in general, point to the same conclusion as was drawn above, namely, that following the application of pollen of an unrelated individual to a given plant the pollen tubes advance farther into the ovary than if the plant in question is self-pollinated. It will be noted, however, that for plant E, the difference, while very small, is in favor of the reverse relation.

With minor exceptions, the average length of the longest pollen tubes is greater at 48 hours than at 30 hours, following both self- and cross-pollination. The average increase in length in the two series during this interval is 1.5 and 1.3 ovule units, respectively. It is not known whether the tubes continue to elongate beyond this period, for at the time of the next collection, 72 hours after pollination, they have disappeared. At 48 hours in the cross-pollinated series, the average lengths of the longest tubes indicate that the latter are already at o near the base of the ovarian cavity. In the selfed series, on the other hand, they are only slightly beyond the midpoint. Even if it be assumed that the pollen tubes in the selfed pistils continue to grow at the same rate during the 24-hour interval after 48 hours as in the preceding 18-hour interval it is evident that in the case of plants

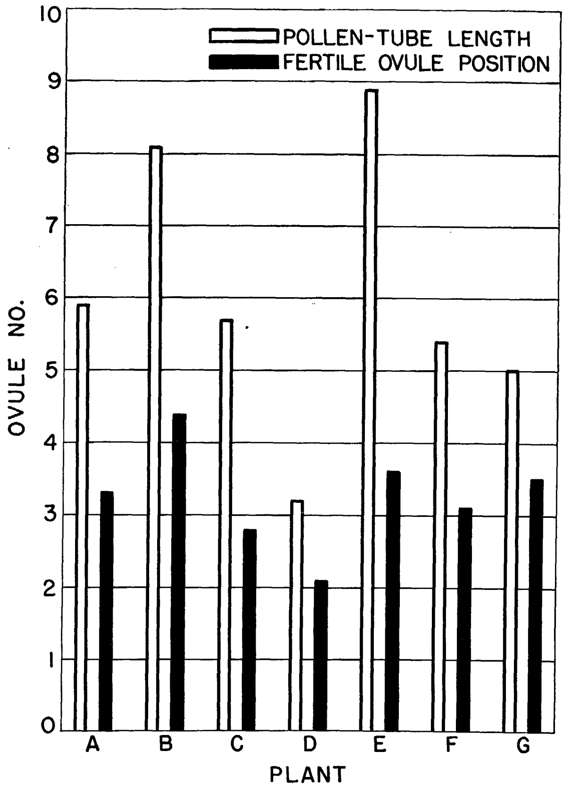


FIGURE 3.—Mean length unit of the longest pollen tubes at 48 hours after pollination in relation to mean position of the fertile ovules following selfing.

A, C, D, F, and G, at least, they would still fall short of reaching the basal ovules.

The mean length of the longest pollen tubes in the selfed series at 48 hours varies from 3.2 ovule units for plant D to 8.9 ovule units for plant E. It is of interest to know whether there is a parallel difference in the distribution of fertile ovules as determined from the collections made at, and following, 72 hours. The mean position of the fertile ovules in each plant has therefore been computed, and the values are presented in table 8 and figure 3 in relation to the corresponding data for pollen-tube length. If the seven plants are now ranked in ascending order of magnitude for mean length of the longest pollen tubes, and in terms of mean position of the fertile ovules, the arrays shown in table 9 are obtained.

TABLE 8.—*Mean length of longest pollen tubes at 48 hours in relation to mean fertile ovule position at 48, 72, 120, and 144 hours after self-pollination*

Plant selfed	Mean length of longest pollen tubes	Mean position of fertile ovule	Plant selfed	Mean length of longest pollen tubes	Mean position of fertile ovule
A	5.9	3.3	E	8.9	3.6
B	8.1	4.4	F	5.4	3.1
C	5.7	2.8	G	5.0	3.4
D	3.2	2.1			

TABLE 9.—*Array obtained when the seven plants are ranked in ascending order of magnitude for length of pollen tubes and position of fertile ovules*

Character	1	2	3	4	5	6	7
Mean length of longest pollen tube	D	G	F	C	A	B	E
Mean position of fertile ovules	D	C	F	A	G	E	B

Several of the differences upon which the order depends are small and of doubtful statistical significance. Considering all the data, however, a positive relation is suggested between the two sets of observations.

Ovules which are not reached by pollen tubes after selfing obviously cannot become fertile. It does not necessarily follow, however, that the converse relation holds, namely, that ovules to which pollen tubes extend usually become fertile. There is little known concerning this phase of the self-incompatibility problem, so that the present data from alfalfa bearing on the question are of much interest.

It may be computed from the data in table 7 that at 48 hours after self-pollination the longest pollen tubes have reached at least to the apical ovule in 98.5 percent of the 66 pistils examined. The longest pollen tubes likewise extend to the second ovule, at least, in 94 percent of the cases. In other words, 48 hours after self-pollination the two ovules at the apical end of the ovary are reached by at least the longest pollen tubes in nearly all of the pistils. The percentages of fertile ovules in these two positions, on the other hand, are much lower. The value for position 1, as may be seen in table 6, is 34.1 percent, and that for position 2 is 27.3 percent.

In the cross-pollinated series at 48 hours the tips of the longest pollen tubes have passed the two apical ovules in all 45 pistils studied. In marked contrast with the low values after selfing, the percentages of fertile ovules in positions 1 and 2 in this group are 86.2 and 84.0, respectively. If it is shown that the two latter values are significantly higher than the corresponding percentages in the selfed series the operation of a factor affecting fertility in addition to pollen-tube length will have been demonstrated.

TABLE 10.—*Summary of analysis of variance in terms of θ values for fertility of the first two ovules at the apex of the ovary following self- and cross-pollination*

Item	Ovule No. 1		Ovule No. 2	
	Self-pollination	Cross-pollination	Self-pollination	Cross-pollination
Weighted mean of θ's	0.620	1.221	0.536	1.177
Variance (6 degrees of freedom)	.414081	1.026656	.823016	.561734
Ovules, number	205	181	205	181
F ($F_{.05}$=4.28)	2.479	1.465	1.684	2.470
Pooled variance (12 degrees of freedom)	.720368	.692374	.733988	.433804
Standard deviation	.849	.832	.857	.659
Standard deviation of difference of 2 means (12 degrees of freedom)	.087	.085	.087	.067
t ($t_{.01}$=3.055)	6.908	7.541	5.552	8.567

In table 10 are summarized the results of the statistical analysis, the latter again being made on the variates transformed in terms of θ. Since both the t values are much larger than the 1-percent value it is inferred that there is a significantly higher ovule fertility associated with cross-pollination than with self-pollination in each of the first two positions in the ovary. Evidently pollen tubes which reach an ovule after selfing frequently fail to effect fertilization. This conclusion has been confirmed by direct observation of pollen tubes within the ovules after the two types of mating. There is evidence of marked stimulation of the tubes in the vicinity of a micropyle after crossing (pl. 1, *C*). Following self-pollination, on the other hand, pollen tubes frequently pass directly by the micropyle of an infertile ovule without showing any tendency to enter (pl. 1, *B*).

COLLAPSE OF FERTILE OVULES

The development of the fertile ovules arising from the two series of matings was followed up to 144 hours after pollination. The average number of cells in the hybrid embryos (including the suspensor) at this stage is about 18.0 and, in the inbred embryos, about 14.5. Not all the ovules in which development is initiated by fertilization, however, continue growth, even during the comparatively brief period of 6 days after pollination. One of the most significant facts the present investigation has brought to light is that the mortality of the ovules containing inbred embryos and endosperms is much higher than that of the ovules containing hybrid embryos and endosperms. It turns out, therefore, that not only are fewer eggs fertilized following self-pollination but the relative fertility associated with this type of mating is further lowered by the collapse of many of the ovules in which development starts.

The data on frequency of collapsing fertile ovules in the two series as found in the pistils collected at 72, 96, 120, and 144 hours after pollination are summarized in table 11 and figure 4. The means by which this class of ovules may be distinguished have been described earlier by Cooper, Brink, and Albrecht (*6*).

Of the 314 fertile ovules observed in the selfed series, 108, or 34.4 percent, were found to be collapsing within 6 days after pollination. The frequencies for individual plants vary from 20.5 percent for E to 51.4 percent for B. In the cross-pollinated series, on the other hand, only 7.1 percent of the 1,211 fertile ovules recorded were collapsing.

TABLE 11.—*Frequency of fertile ovules collapsing in seven plants after self- and cross-pollination*

[Data based on collections at 72, 96, 120, and 144 hours after pollination]

Plant	Fertile ovules: Total	Fertile ovules: Collapsing		θ
	Number	*Number*	*Percent*	
Selfed:				
A	37	9	24.3	0.515
B	37	19	51.4	.799
C	20	7	35.0	.633
D	17	7	41.2	.697
E	39	8	20.5	.470
F	109	39	35.8	.641
G	55	19	34.5	.628
Total	314	108	34.4	
Crossed:				
A × B	187	13	7.0	.268
B × C	110	5	4.5	.214
C × D	171	13	7.6	.279
D × E	171	16	9.4	.312
E × A	146	9	6.2	.252
F × G	228	14	6.1	.250
G × F	198	16	8.1	.289
Total	1,211	86	7.1	

Furthermore, the percentage of aborting ovules of this class is uniformly low in all the plants. The difference in the mean values is highly significant statistically as the computations in the following paragraph show.

The weighted mean θ for the self-pollinated series is 0.624, based on

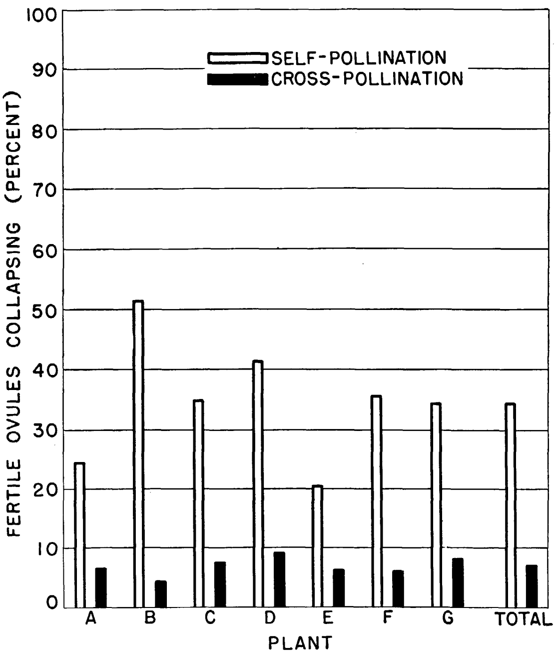


FIGURE 4.—Percentage of fertile ovules collapsing during the first 144 hours after self- and cross-pollination.

314 ovules, and 0.269 for the cross-pollinated group, based on 1,211 ovules. The standard deviation of the difference between the means of the two groups is 0.034. It follows that $t=10.44$, which is much larger than the value of t at the 1-percent point, namely, 3.055.

The relation between position in the ovary and frequency of collapse of fertile ovules is shown in table 12. It will be noted that, after selfing, the proportion of ovules in which development is arrested is comparatively high throughout. There is probably a tendence, however, for the frequency to increase somewhat in the lower positions. In the cross-pollinated series the values are fairly constant down to the ninth ovule, after which a small increase is shown. Uniform gradients similar to those associated with fertilization in the two series are not in evidence here.

TABLE 12.—*Frequency of ovules collapsing in relation to position in the ovary following self- and cross-pollination*

[Data based on collections at 72, 96, 120, and 144 hours after pollination]

Ovule No. [1]	Self-pollination			Cross-pollination		
	Fertile ovules			Fertile ovules		
	Total	Collapsed		Total	Collapsed	
	Number	*Number*	*Percent*	*Number*	*Number*	*Percent*
1	70	18	25.7	156	12	7.7
2	56	16	28.6	152	13	8.5
3	59	17	28.8	133	3	2.2
4	49	20	40.8	139	11	7.9
5	33	12	36.4	131	12	9.2
6	21	10	47.6	125	10	8.0
7	13	10	76.9	111	7	6.3
8	9	4	44.4	90	3	3.3
9	3	1	33.3	80	3	3.7
10	1	0	0.	58	7	12.1
11				28	3	10.7
12				8	2	25.0
Total	314	108	34.4	1,211	86	7.1

[1] The apical ovule is No. 1.

FREQUENCY OF FERTILIZATION AND COLLAPSE OF FERTILE OVULES IN RELATION TO THE PRODUCTION OF MATURE SEEDS

The seven plants used in the experiment formed about 10.5 ovules per pistil, on an average (table 6). It is shown in table 1 that, per flower pollinated, 4.5 mature seeds were formed after crossing and 0.8 seed after selfing. To what extent is the difference between the potential and the actual fertility accounted for by the two factors measured, namely, failure of fertilization and collapse of fertile ovules?

Referring to table 2, it may be seen that 14.6 percent of the ovules became fertile after selfing and 66.2 percent after crossing. Of the ovules which became fertile 34.4 percent containing inbred embryos and endosperms collapsed within 6 days after pollination as compared with 7.1 percent containing hybrid embryos and endosperms (table 11). Considering these two factors alone, the expected average number of seeds per flower is 1.00 and 6.15 for the two respective types of mating (fig. 5). This means that under the conditions of the present experiment about 98 percent of the difference between the potential and actual fertility following self-pollination and about 67 percent following cross-pollination are accounted for by the lack of fertilization and the collapse of fertile ovules during the first 6 days.

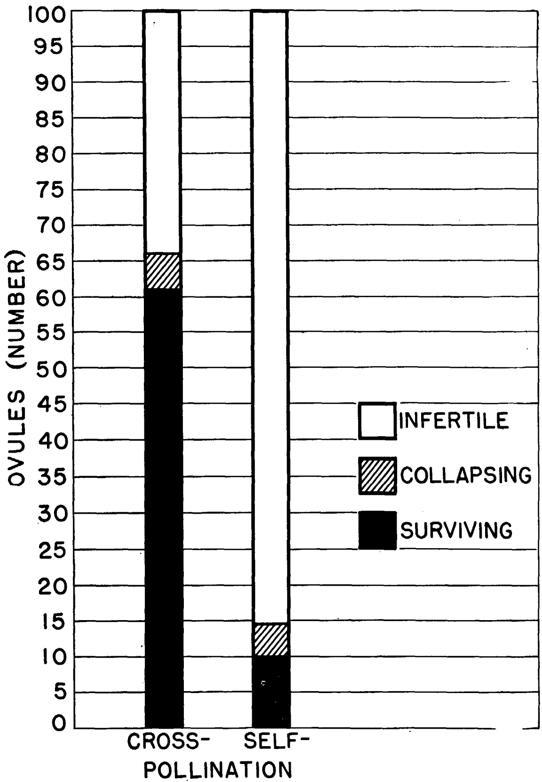


FIGURE 5.—Effect on fertility of failure of fertilization and the collapse of fertile ovules during the first 144 hours after pollination, following selfing and crossing.

DISCUSSION

In an earlier study of fertility in alfalfa in which the effects of self-pollination alone were determined, Cooper, Brink, and Albrecht (*6*) observed that (1) the pollen tubes frequently fail to reach the basal ovules, (2) many ovules remain infertile even though pollen tubes are present, and (3) abortion of fertile ovules is of common occurrence. These findings, which are confirmed in the present investigation, assume added significance in the light of the important additional fact now established that if cross-pollination to an unrelated individual is substituted for selfing the fertility is greatly increased. Crossing was found to raise the proportion of ovules becoming fertile from 15 percent to 66 percent, on an average, and to reduce the abortion of fertile ovules during the first 144 hours after pollination from 34 percent to 7 percent. The higher ovule fertility is found to result from more extensive growth of the pollen tubes within the cavity of the ovary and an increased tendency for the pollen tubes which reach the ovules to enter the micropyles and accomplish fertilization. These facts make it clear that one of the basic phenomena involved in reproduction in alfalfa is partial self-incompatibility.

About five and one-half times as many mature seeds were formed per flower pollinated after crossing as after selfing in the seven plants used in the present experiment. On the basis of the values given in the preceding paragraph for frequency of fertilization and survival of fertile ovules up to 144 hours after pollination, crossing would be expected to increase the fertility about sixfold. Some additional ovules probably collapse after 144 hours, so that an exact correspondence between the two ratios is not to be anticipated. The fact, however, that the values are of the same order of magnitude lends further weight to the conclusion that the two factors identified are mainly responsible for the increased seed formation on cross-pollination.

Significant differences between individuals were found in the proportion of ovules becoming fertile after self-pollination. The extreme values within the seven plants examined were 6.7 and 21.5 percent. In a previous investigation, Cooper, Brink, and Albrecht (*6*) found one plant in which 43.7 percent of the ovules became fertile after selfing. Doubtless study of larger populations would disclose an even wider spread. The conclusions that self-incompatibility in alfalfa is only partial and varies considerably from plant to plant are in accord with the writers' observations on fertility in larger numbers of individuals being selfed in a breeding experiment by hand-tripping of the flowers. Rarely is a plant found on which no seed is formed after selfing, although the yield is often low. Occasional individuals occur in which the self-fertility is relatively high. It is not known in these latter cases whether it could be increased by applying pollen from unrelated plants. All seven plants in the present experiment, however, showed a statistically significant increase in percentage of fertile ovules on crossing in spite of considerable variation in degree of self-incompatibility.

The abortion of fertile ovules, which occurs so freely following self-pollination, may be a manifestation of self-incompatibility per se, or it may be an inbreeding effect. That is to say, either some of the genes conditioning pollen-tube behavior also may tend to cause ovule collapse, or recessive genes at other loci, adversely affecting early development of the zygote and brought together in homozygous condition by the inbreeding, may be responsible. When the data in table 2 showing the percentage of ovules becoming fertile after self-pollination in the seven plants are compared with those in table 11 showing the frequency of fertile ovules collapsing in the same individuals, no correlation is apparent between the two sets of values. Examination of the data obtained by Cooper, Brink, and Albrecht (*6*) on another series of alfalfa plants leads to the same result. While the evidence is rather limited, it indicates that the collapse of fertile ovules after selfing is more likely an effect of inbreeding than a direct manifestation of self-incompatibility.

The fact is well known to those who have had an opportunity to observe it that alfalfa is highly variable in reproductive behavior. The plant, although blossoming freely, rarely sets seed in many geographical areas in which it is satisfactorily grown as a forage crop. Even in regions where seed is produced commercially, the yield may fluctuate widely. The reasons for the variability are not yet sufficiently well understood to permit of a general interpretation of this behavior. Meanwhile, however, the implications of partial self-incompatibility may be examined.

The following two deductions of direct significance for an understanding of seed formation in alfalfa may be drawn from the evidence presented above: (1) The potential fertility of a plant will usually, if not always, fall far short of complete realization if self-pollination alone prevails. Under uniform environmental conditions the degree of self-incompatibility varies widely from plant to plant in alfalfa, as it does in many other self-incompatible species (*11*). The fertility of the ovules was markedly higher following cross-pollination, however, in each of the cases in which a direct comparison was made in the present study. The degree of self-incompatibility of a given genotype may also vary according to external conditions judging from the behavior of other self-incompatible species (*11*); but whether the environment can ever shift the reaction of an alfalfa plant to its own pollen so that the usual advantage of crossing disappears remains to be shown.

(2) If, on the stigmas of a given plant, the latter's own pollen is present along with that from an unrelated plant, under conditions favorable for germination, the pollen from the unrelated plant will probably have a large advantage in effecting fertilization. This point is of particular significance for alfalfa since in conformity with the structure of the flower, the stigma usually receives a copious amount of pollen from the anthers encircling it even before the flower opens. The validity of this conclusion rests on (a) the observed fact that, following crossing, the pollen tubes not only grow more rapidly and extend farther into the ovarian cavity than after selfing but they also show a greater tendency to enter the micropyles and effect fertilization in the ovules reached and on (b) the applicability to alfalfa of the rule East and Park (*7*) established for self-incompatible tobaccos that mixed self- and cross-pollination does not increase self-fertilization.

The discovery of partial self-incompatibility brings into sharp relief another phase of the fertility problem in alfalfa, namely, the means by which pollen produced by one plant gains access to the stigma of another. Carlson (*5*) and Hadfield and Calder (*9*) have observed pollen on the standards of untripped flowers in the field. Doubtless much of this is brought from other plants by air currents and insects. When a flower trips, some of the pollen on the standard may be captured by the stigma and cross-pollination thus be effected. The question becomes perplexing, however, when regard is given to the findings of Carlson (*5*) that, under Utah conditions, alfalfa commonly sets seed without tripping of the flowers. Brink and Cooper (*3*) found the same conditions prevailing under unusually hot and dry weather at Madison, Wis., in 1936. Possibily the degree of self-incompatibility is lowered by the conditions of growth in these cases and much self-fertilization occurs. It seems more probable, however, that pollen from other plants gains access to the stigmas of untripped flowers by some means not yet described. Brink and Cooper (*3*) observed pollen germinating within untripped flowers and pollen tubes entering the styles, even in the late bud stage. Almost certainly the pollen functioning in the latter cases was from the same respective flowers. Conceivably, this early self-pollination initiates wilting of the corolla and stimulates development of the pistil so that the stigma becomes exposed to pollen from other flowers. Since after selfing, the pollen tubes grow more slowly and fail to fertilize the eggs in many of the ovules, the pollen from other individuals might largely prevail in fertilization in spite of its later start. Obviously, this question requires further investigation.

SUMMARY

Following self-pollination under greenhouse conditions only 14.6 percent of the ovules of alfalfa become fertile, on an average, as compared with 66.2 percent after cross-pollination on the same plants.

Individuals vary significantly in the proportion of ovules that become fertile after selfing. The variability is less after cross-pollination.

Most of the fertile ovules resulting from self-pollination are in the apical half of the ovary. A rather uniform gradient in fertility occurs from the apex downwards, the ovules in the lowermost positions rarely containing fertilized eggs.

A parallel gradient in fertility is found after cross-pollination, although the proportion of ovules becoming fertile in each position is much higher than after selfing.

Pollen-tube growth, as determined by the position of the tips of the longest pollen tubes at 30 hours and at 48 hours after pollination, is more rapid in cross matings than after selfing.

It is probable that the basal ovules in the ovary are seldom reached by pollen tubes after selfing. Following cross-pollination the tubes usually grow to the base of the ovary.

In the self-pollinated series there appears to be a positive, although not high, correlation between mean length of the longest pollen tubes and mean position of the fertile ovules from plant to plant.

The low fertility of the ovules following self-pollination is due only in part to restricted pollen-tube growth. Direct observation confirms other, more general, evidence adduced that frequently after selfing the pollen tubes pass directly by the micropyles of ovules containing unfertilized eggs.

About one-third of the fertile ovules in the selfed series were found to be collapsing in the pistils collected at 72 to 144 hours after pollination as against only 7.1 percent in the crossed series.

Taking into consideration the differentials both in proportion of ovules becoming fertile and proportion of fertile ovules collapsing, the net fertility at 144 hours after pollination is about six times as high in the crossed series as in the selfed series.

The partial incompatibility associated with self-pollination and the high incidence of abortion of ovules containing inbred embryos during the first 6 days after pollination appears sufficient to account for most of the difference in seed production following selfing and crossing.

Under the conditions of the present experiment about 98 percent of the difference between the potential and actual fertility following self-pollination and about 67 percent following cross-pollination are accounted for by the frequency of fertilization and the collapse of fertile ovules during the first 6 days.

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