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Time of Cross- and Self-pollination Affects the Amount of Self-seed Set by Partially Self-incompatible Plants of *Brassica oleracea*

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Summary. The relative ability of cross- and self-pollen to achieve fertilisation in *Brassica* was studied by making double pollinations using cross-pollen carrying a dominant seedling marker gene. With simultaneous self- and cross-pollination 12-40% self-seed was set, but when cross-pollen was applied to the stigma four hours before self-pollen, only 2-4% self-seed was obtained. In two plants to which cross-pollen was applied at various time intervals after self-pollen there was a tendency for the percentage of self-seed to increase as the time interval increased. In a third plant this trend was not apparent, probably because of a greater degree of self-incompatibility. The competitive advantage of the first pollen to arrive on the stigma is discussed in relation to the strength of the self-incompatibility and the sib problem in F_1 hybrid brassicas.

Key words: Self-incompatibility – Time of pollination – Pollen tube Competition – Seedling markers

Introduction

There was no significant difference in the growth rates of self- and cross-pollen in styles of *Brassica oleracea* at 23° C (Ockendon & Gates 1975). This suggests that those self-pollen tubes which succeed in penetrating the stigmatic surface probably compete equally with cross-pollen tubes in fertilisation. Clearly, the number of self-pollen tubes penetrating the stigma is related to the strength of the self-incompatibility reaction. In plants which are very highly self-incompatible the very few self-pollen tubes which might be present in the style would be greatly outnumbered by cross-pollen tubes, assuming both types of pollen had reached the stigma. Thus it is desirable that the inbred lines of *Brassica* being used to make F_1 hybrid cultivars should be very highly self-incompatible. However, this is often not the case as indicated by the unacceptably high levels of sibs in many F_1 hybrid seed lots, especially those of Brussels sprouts (Johnson 1972).

In plants which are not completely self-incompatible, self-pollen tubes would be expected to have an initial advantage over cross-pollen tubes if the self-pollen arrived on the stigma before the cross-pollen. The extent of this advantage would depend not only on the time interval between the arrival of the two kinds of pollen, but also on the number of tubes of each type in the style. As there is no simple technique for distinguishing self- and cross-tubes growing side by side in a style, competition between them can best be studied by obtaining seed from mixed or double pollinations. Seedlings produced by such seed can then be scored as cross or self, provided the cross-pollen carries a suitable seedling marker gene.

To investigate the effect of time of arrival on the stigma of cross- and self-pollen, a series of pollinations was made in which the two types of pollen were applied at the same time or with various time intervals between the two applications. The cross-pollen carried a dominant seedling marker gene; the two markers used were one for anthocyanin pigmentation and the other for hairiness. The plants used as female parents were moderately or weakly self-incompatible, thus ensuring a reasonable number of self-pollen tubes in the style after self-pollination.

Material and Methods

The plant (WC 15/1) used as a female in 1975 was a hairy wild cabbage (*Brassica villosa* Biv.) derived from material collected in Sicily. This plant was homozygous for *S*5, one of the weakest *S*-alleles in *B. oleracea* L. (Thompson and Taylor 1966). The plants used in 1976 were homozygous for *S*15, also a very weak *S*-allele, and were derived from a cross between marrow-stem kale (*B. oleracea* var *acephala*) and Brussels sprouts (*B. oleracea* var *gemmifera*). All plants used as females showed marked self-compatibility as assessed by pollen tube counts.

Cross-pollen carrying a dominant marker gene was obtained either from red Brussels sprouts cv 'Rubine' or from hairy wild cabbages. Although the wild cabbages were classified as *B. villosa*, they were completely interfertile with all the cultivated forms of *B. oleracea* with which they were tested.

The plants were grown in pots in an insect-proof glasshouse. Each experiment was performed on a different day during the winter and early spring, and in 1976 supplementary lighting was provided.

Young flowers were emasculated, generally on the day prior to pollination, and the stigmas were carefully inspected to ensure that they were completely free of self-pollen. Two methods of pollination were used. In the first set of experiments (A-G, Table 1) two successive layers of pollen were applied to the whole of the stigma surface with a small sable brush. In the second set of experiments (H-Q, Table 1), the self-pollen was applied to one-half of the stigma and the cross-pollen to the other. The central groove of the stigma was used as a dividing line, and pollen was applied to each half of the stigma with flat-ended sable brushes. The order in which the two kinds of pollen were applied could be important in the whole-stigma pollinations but would be unlikely to have any effect with the half-stigma method.

Both types of pollen were applied on the same day except where self-pollen was applied 16 hours before cross-pollen; self-pollen was then applied in the evening, and cross-pollen the following morning.

In each experiment 8 or 10 flowers received double pollination, two flowers received self-pollen alone and two received cross-pollen alone. In 1975 pollen tube counts were made on the flowers being kept for seed. The styles were excised two days after pollination using the method described by Gates and Ockendon (1975). In 1976 two additional flowers were cross-pollinated and four selfed in each experiment solely for pollen tube counts. The counts on these flowers were made either one or three days after pollination. The styles were prepared for examination by the decolourised Aniline Blue method (Kho and Baër 1968) and the pollen tube counts made as near to the tip of the style as possible.

Each plant was bud-selfed so that known self-seedlings were available for comparison with the seedlings from double pollinations. Seedling assessments involving the anthocyanin marker character were made after growing the seedlings to a height of 2-4 cm in petri-dishes under normal indoor lighting conditions. The seedlings were somewhat etiolated but the differences between cross- and self-seedlings were best expressed under these conditions (Attia and Munger 1950). Seed lots containing the hairy marker character were sown in trays of Levington compost in a glasshouse and scored when the seedlings had one or two true leaves. The petioles of the true leaves of self-seedlings were either entirely glabrous or very sparsely hairy whereas those of the cross-seedlings were extremely hairy.

Results

Seed set was generally good, few pods having less than 10 seeds. Seed germination was at least 90% in most cases. In experiments A-D (Table 1) using the anthocyanin marker and experiments M-Q using the hairy marker, there was little difficulty in distinguishing between cross- and self-seedlings. However, in experiments E-L there was difficulty with the use of the anthocyanin marker because of variability of its expression. Some of the known selfed-seedlings from *S*15 plants, which were expected to be completely green, showed traces of anthocyanin. Moreover, among known cross-seedlings, which should all have been purple, as many as 27% were initially scored as green. However, more detailed inspection revealed three types of seedlings. In addition to those that were distinctly purple, a light purple type was recognised in which the green cotyledons had a purplish tinge on their underside and distinctly purple mid-veins. The green seedlings were totally devoid of anthocyanin apart from a purplish

Table 1. Amount of self-seed resulting from separate application of self-(s) and cross-(c) pollen to a single stigma. In experiments A-L the cross pollen came from a red Brussels sprout and in M-Q from a hairy wild cabbage. In experiments A-G the whole stigma was pollinated with both kinds of pollen, whereas in the rest, each kind of pollen was applied to one half of the stigma only

Experiment	Female parent	First application	Time interval hours	Second application	No. pods	No. seedlings scored	% Self seedlings
A	WC 15/1	C	4	S	7	109	4.5
B	WC 15/1	S	0	C	7	90	40.0
C	WC 15/1	S	6	C	8	118	33.8
D	WC 15/1	S	8	C	8	129	92.2
E	*S* 15/15	S	0	C	10	205	27.3
F	*S* 15/15	S	0	C	10	280	16.8
G	*S* 15/15	C	0	S	9	210	13.3
H	*S* 15/15	S	0	C	10	283	12.4
I	*S* 15/13	S	0	C	10	345	16.8
J	*S* 15/13	S	4	C	10	358	31.8
K	*S* 15/13	S	8	C	10	336	28.3
L	*S* 15/15	S	16	C	10	274	19.3
M	*S* 15/14	C	4	S	10	326	2.5
N	*S* 15/14	S	0	C	10	303	5.9
P	*S* 15/14	S	4	C	10	247	26.9
Q	*S* 15/14	S	16	C	10	324	99.4

tinge to the upper hypocotyl. The light purple seedlings were considered to have resulted from cross-fertilisation; some such seedlings may have been mis-classified as green before the light purple type was recognised. Difficulties encountered with the anthocyanin marker illustrate the need to have control seedlings which are known to be either cross- or self- when a mixed batch of seedlings is being scored. As a few of the seedlings in experiments E-L which were scored as green should probably have been scored as purple, the figures for the percentage self-seedlings for experiments E-L (Table 1) are probably slight over-estimates.

In some cases the variation between flowers within an experiment was quite small. For instance, in experiment H the number of seeds per pod varied from 24 to 34 and the percentage self-seed per pod was 7-18%. However, in other cases the flower to flower variation was much greater, the most extreme case being experiment C where the number of seeds per pod was 5-23 and the percentage self-seed varied from 0-92%. The results of chi-squared tests showed that there was more flower to flower variability in the percentage self-seed set than would be expected by chance. However, the variability tended to be lowest when the mean percentage of self-seeds was about 10-20%.

The results in Table 1 may be considered in three groups, according to whether the cross-pollen was applied before, simultaneously with or after the self-pollen. In experiments A and M cross-pollen was applied four hours before the self-pollen and very small percentages of self-seed were obtained. This indicated that self-pollen still competes to some extent with cross-pollen, even when it arrives on the stigma four hours after the cross-pollen.

When pollinated simultaneously with self- and cross-pollen the WC 15 plant gave 40% self-seed (experiment B); the *S*15 plants gave 12-27% self-seed (experiments E-I). Both seedling and pollen tube counts (Table 2) indicated that WC 15 was somewhat more self-compatible than the *S*15 plants. The same pollination method was used in experiments E and F but the percentage self-seedlings was significantly different at the 1% level. There is no obvious explanation for this, but it may have arisen because of difficulties in classifying the seedlings as cross or self. The amounts of selfing in experiments F-I were very similar although different pollination methods were used. Cross-pollen was applied immediately before self-pollen in experiment G, and the reverse was true for experiment F, but this made no significant difference to the percentage self-seed set. Although the pollen which arrives on the stigma first may be more favourably placed for germination than pollen arriving later, this advantage, if any, would seem to be small.

When self-pollen was applied six hours before cross-pollen (experiment C) there was no increase in the amount of self-seed compared with the simultaneous pollination (experiment B), but when the time interval was increased to eight hours (experiment D) there was a great increase in the amount of self-seed (Table 3). The percentage of self-seed obtained in experiment C was lower than expected. The figure obtained is perhaps rather unreliable because of the unusually high flower to flower variation in

Table 2. Pollen tubes and seed-set resulting from pollination with self- or cross-pollen alone. Each figure is the mean for two flowers, except the figures for self-tubes in experiments E-Q which are based on four flowers each. Night temperatures are given only where the pollen tube counts were made within 24 h of pollination

Experiment	Time of pollen tube count (days)	Minimum night temperature °C	Self-pollen tubes	Self seeds	Cross-pollen tubes	Cross Seeds	Female parent
A	2	–	75	8	>80	11.5	WC 15/1
B	2	–	>80	19	>80	14	WC 15/1
C	2	–	>80	16.5	33	6	WC 15/1
D	2	–	40	12.5	>80	11.5	WC 15/1
E	1	13	0	16.5	>80	21.5	S 15/15
F	3	–	37	27.5	63	29	S 15/15
G	3	–	29	21	>80	23	S 15/15
H	1	13	8	19.5	>80	28.5	S 15/15
I	1	9	2	30.5	>80	36	S 15/13
J	1	16	30	34.5	>80	–	S 15/13
K	3	–	77	32	>80	31	S 15/13
L	1	5	2	21.5	>80	31.5	S 15/15
M	3	–	>80	33	50	32.5	S 15/14
N	1	14	25	26	>80	28	S 15/14
P	3	–	25	28.5	49	26	S 15/14
Q	1	15	60	32	>80	29.5	S 15/14

Table 3. Effect of various time intervals on the percentage self-seed resulting from double pollinations using cross (C) and self (S) pollen. The letters in brackets refer to the experiments listed in Table 1 from which the data has been taken and rearranged for ease of reference

Pollination treatment (time interval in hours)			Female parent		
			WC 15/1	*S* 15/13 (or /15)	*S* 15/14
C	4	S	4 (A)	–	2 (M)
S	0	C	40 (B)	17 (I)	6 (N)
S	4 or 6	C	34 (C)	32 (J)	27 (P)
S	8	C	92 (D)	28 (K)	–
S	16	C	–	19 (L)	99 (Q)

this experiment already mentioned. A similar trend, with increasing amounts of self-seed as the time interval between the self- and cross-pollen applications was increased, was evident in experiments N-Q. In experiment Q where the cross-pollen was not applied until 16 hours after the self-, there was virtually no cross-seed set. However, this trend was not shown in experiments I-L (Table 3). In experiment L, where the percentage self-seed was much lower than expected, the 16 hour period in which the self-pollen tubes were not competing with cross-tubes occurred during a night when the temperature was low (minimum 5° C). It seems probable that the higher self-incompatibility of the *S*15/15 plant on this occasion inhibited most of the self-pollen tubes and allowed the cross-tubes to overtake them.

In most cases, almost full seed set was obtained from single pollinations whether using self- or cross-pollen (Table 2). The WC 15 plant gave higher and more consistent numbers of self-pollen tubes than the *S*15 plants. The latter gave high seed sets after self-pollination, but the number of self-pollen tubes counted varied greatly and was sometimes very low. This was particularly true of experiment L (Table 2) which had a mean of only 2 pollen tubes per flower. This was associated with a low night temperature of 5° C. Although the number of self-pollen tubes counted was very low, the self-pollinated flowers kept for seed had a mean of 21.5 seeds per pod. However, the pollen tube counts were made within 24 hours of pollination, whereas any tubes which managed to penetrate the stigma before it withered (3-4 days) would be potentially capable of effecting fertilisation and producing self-seeds. Counts made after 3 days (Table 2) gave pollen tube numbers of 25 or more. This is an interesting example of the interaction between incompatibility and pollen availability. The incompatibility reaction in experiment L was strong enough to give very much less self-seed than cross although the cross-pollen was competing at a 16 hour disadvantage with the self-pollen, but was not strong enough to prevent good self-seed set when there was no competition from cross-pollen.

Discussion

The ability of cross- and self-pollen tubes to effect fertilisation when they are competing with one another in *Brassica* carpels may be affected by a number of factors. These include the viability of both the cross- and self-pollen, the competitive ability of tubes produced by pollen from different plants, the degree of self-incompatibility and the seed producing capacity of each flower. The viability of the pollen used in our experiments was not tested as such, but the fact that high numbers of seeds were set from both the self- and cross-pollinations (Table 2) indicates that none of the pollen used had markedly reduced viability.

Although no difference was found at 23° C between the growth rates of cross- and self-pollen tubes in *Brassica* styles (Ockendon and Gates 1975), it is not certain that this would be true at the lower temperatures prevailing in the experiments reported here. Irrespective of whether the pollen is cross- or self-, pollen from different sources may have different rates of tube growth. The fertilising ability of pollen grains, which depends in part on the rate of pollen tube growth, was studied in maize by Pfahler (1965), using mixtures of cross- and self-pollen and scoring the kernels produced. He used pollen from four sources and found a range of self-fertilisation varying only from 33 to 43%. Very little evidence on this subject is available for *Brassica*, but Watts (1963) found that cauliflower pollen had no consistent advantage over Brussels sprout pollen when growing in cauliflower carpels.

Variation in the level of self-incompatibility of the plants tested has already been mentioned, and was more apparent in the pollen tube counts than in the seed-set data (Table 2). In general, variation in the seed-producing capacity of individual flowers was no greater than might have been expected had all the flowers been simply cross-pollinated. Hence the main uncontrolled factor affecting our results was probably variation in the strength of the incompatibility reaction of the plants used. Although the flowers used were as uniform as possible, there was still flower-to-flower variation in self-incompatibility which is very difficult to reduce. The plant-to-plant variation in this factor could have been reduced if it had been possible to control the temperature and to perform some of the experiments simultaneously.

Our results show that in plants with fairly weak self-incompatibility, the relative time of arrival of self- and cross-pollen on the stigma can greatly affect the amount of outcrossing which occurs. It is difficult to estimate how much time self-pollen must have in order to have a clear advantage over cross-pollen. In our results, the shortest time interval to give a large effect was 8 hours, but shorter time intervals may have an effect at higher temperatures.

For the production of F_1 hybrid *Brassica* crops it is desirable to have inbreds which are as highly self-incompatible as possible to minimise the occurrence of sibs. However, the inbreds with the best agronomic characters are very seldom those with the highest levels of self-incompatibility. The two Brussels sprouts inbreds used by Johnson (1966) were partially self-compatible, but not quite as self-compatible as the plants used by us. Johnson (1966) and Johnson and Morgan (1969) found that the sib-rate for a particular F_1 hybrid varied from year to year and from site to site. Faulkner (1974) found that bees pollinating crops of Brussels sprouts grown for F_1 hybrid seed production were very selective in their behaviour, thereby ensuring much more self- and sib-pollination than cross-pollination. Because of pollination by wind or agitation (Watts 1963) and the behaviour of pollinating insects, stigmas are likely to receive self-pollen before cross-pollen. If cross-pollen arrives on the stigmas within an hour or two of the self-pollen it is unlikely that much self-seed will be set unless the plants are markedly self-compatible. However, if cross-pollen does not reach the stigma until eight hours or more after self-pollen, substantial quantities of self-seed may be set, unless the female plants are consistently highly self-incompatible. In view of the potential advantage to self-pollen which arrives on the stigma before cross-pollen, it is desirable that any delay in the arrival of the cross-pollen should be minimised by maintaining a high population of pollinating insects. Evidence also suggests that low temperatures at the time of pollination will tend to favour cross-pollen and delay arrival of self-pollen tubes at the ovules.

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