Theor Appl Genet (1993) 85:755–760

THEORETICAL AND APPLIED GENETICS

Quantitatively determined self-incompatibility. IV. Pollination and seed set in *Borago officinalis*

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Received May 4, 1992; Accepted June 3, 1992
Communicated by G. Wenzel

Summary. The outcomes of sequential double pollination, mixed double pollination and single pollination are compared. Single pollination leads to lower seed set than double pollination. Systematic differences between female genotypes are shown to be possible. It is also shown that failure to set seed is generally due to pre-zygotic maternal factors.

Key words: Pollination – Seed set – Pollen competition – Pre-zygotic factors

Introduction

In 1970 Crowe published a study of crossing in borage, *Borago officinalis*, in which she claimed that borage had a quantitatively determined self-incompatibility system and that this system exerted its effect post-zygotically, through embryo abortion. The discovery of such a system is of great interest from an evolutionary point of view, since it has been proposed that the many different oligogenic systems could have evolved from a more diffuse self-non-self recognition process through the selective enhancement of a few of the elements of such a system. Hence, a multifactorial system might provide insight into such a hypothetical evolution.

We therefore initiated a study of the breeding system of borage. At the same time, we developed two arbitrary theoretical 10-gene model systems in order to determine what some of the properties of such systems might be, and to assess how such systems might differ from the thoroughly investigated oligogenic types. These studies (Mayo and Leach 1989; Leach, Mayo and Bürger 1990; Leach and Mayo 1990) showed that polygenic systems can persist, with similar stability to oligogenic systems but with greater levels of incompatible pollination, and that they should maintain genetical variation at about the same level. Borage was shown to be self-fertile, to manifest inbreeding depression in seed set (the extent varying with the intensity of inbreeding) and to be almost completely invariant.

To complete the study, we needed to evaluate the possibility of pollen competition as a mechanism for ensuring outbreeding and to determine whether embryo abortion actually occurs.

Methods and results

Segregation and seed set

An extensive program of double pollinations, 24 h apart, was carried out to determine whether more than one visit by a pollinator might influence seed set. Field observations indicate that pollinators (mainly bees) work flowers extensively and yield by far the best seed set. These pollinations were made with the two different isozyme variants of Diaphorase (DIA), viz., 3-3 and 4-4, in the following way:

	1st pollination	2nd pollination
plant 3-3 or 4-4	3-3	3-3
	3-3	4-4
	4-4	3-3
	4-4	4-4

also as controls single pollination with only one type

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of pollen and single pollination with mixed pollen (ten crosses were made in each category)

In addition, all seeds set by open pollination during the course of the crossing program were collected. The numbers of seed set are recorded in Table 1. All seeds set by 3-3 then 4-4, 4-4 then 3-3, as well as by open pollinations and mixed pollinations, were grown and the plants typed. The results are presented in Table 2. In only one case was the second pollen effective in fertilization. In a total of 236 seeds set by open pollination only one was clearly due to outcrossing, indicating a rate of $\frac{2}{236} = 8.5 \times 10^{-3}$ outcrossing.

In addition to counting seeds set per capsule, we classified plants setting two seeds (nutlets) as to the position of these seeds, i.e., side-by-side or diagonally opposite. The results are presented in Table 3. All plants are homogeneous ($\chi_7^2 = 2.64$) but the totals do not fit the expected 2:1 ratio ($\chi_1^2 = 16.13$). These plants show a clear bias towards setting seed in the side-by-side position.

Finally, we assessed the extent to which seed number per capsule influenced weight per seed. We analysed the results of the crosses for which seed-set data are presented above. We, therefore, fitted a model that included the number of seeds per capsule (1, 2, 3, 4) and plant identification as female and male parent. Table 4 shows the analysis of variance, and Table 5 the least-square means for seed weight for the significant effects, which were number of seeds per capsule and plant identification as female parent. Not unexpectedly, seed weight depended inversely on the number of seeds per capsule.

Viability also depended on seed set, the coefficient of linear regression of plants raised against seeds per capsule being 0.784 ± 0.031 with 240 degrees of freedom, i.e., a value differing significantly from zero and unity.

Failure to set seed

To investigate the consistent failure to set four seeds in every capsule, given that pollination is sufficient to do so in some cases, we examined the developing ovaries by microscopy. Ovaries were prepared using a modification of the method described by Young, Sherwood and Bashaw (1979) whereby fixed ovaries were cleared with methyl salicylate and observed as whole mounts with interference contrast microscopy.

Flower heads were collected from glasshouse-grown plants and fixed in 3:1 methanol:glacial acetic acid. After 24 h at 4 °C the fixative was changed and after a further 24 h buds were transferred to glass vials containing 100% ethanol and cleared as follows: 1:1 ethanol:methyl salicylate, 100% methyl salicylate. Cleared flowers were stored in methyl salicylate. For microscopic examination, cleared ovaries were dissected from flowers and mounted in methyl salicylate under an unsealed coverslip on a welled slide. The ovules and embryo sacs were viewed using a Reichert Univar microscope fitted with Nomarski optics and photographed with 35 mm Kodak Ektachrome 100 plus film.

Ovules were found to be unitegmic and hemianatropous. The age of the ovules limited the study to observations of mature embryo sacs which were found to have an oblong to elliptical shape and to be surrounded by an endothelium several cells thick. The origin of the endothelium was not observed and its source may be the single integument or the nucellus (Kapil and Tiwari 1978). The bulk of tissue involved

Table 1. Crossing program 11/89

Plant	Seeds scored	Type of pollination							
		3-3 then 4-4		4-4 then 3-3		Open pollination		Mixed pollination	
1-1 (33)[a]	3/3	3 (33)	8/11	8 (34)	8/8	8 (33)	6/9	4 (33)	2 (34)
1-4 (33)	21/23	21 (33)	16/16	16 (34)	20/20	20 (33)	5/5	1 (33)	4 (34)
1-6 (33)	18/18	18 (33)	7/8	7 (34)	41/42	41 (33)	11/12	9 (33)	2 (34)
1-12 (33)	22/24	22 (33)	16/19	16 (34)	7/8	7 (33)	17/21	1 (33)	16 (34)
2-1 (44)	18/21	18 (34)	30/31	27 (44) 3 (34)[b]	64/67	64 (44)	17/21	10 (34)	7 (44)
2-4 (44)	19/20	19 (34)	27/30	27 (44)	64/64	64 (44)	9/10	6 (34)	3 (44)
2-5 (44)	16/17	16 (34)	13/14	13 (44)	16/18	13 (44) 1 (34)[c]	4/12	5 (44)	4 (34)
2-10 (44)	5/12	5 (34)	2/8	2 (44)	24/39	24 (44)	–	–	–

[a] (33) means genotype (3-3), (34) means genotype 3-4, etc

[b] Used 2nd pollen, cf first

[c] Indicating outcrossing

Table 2. Seed set data

Pollination	Plant								
	1-1			1-4			1-6		
	n	%	$\bar{x} \pm$ SE	n	%	$\bar{x} \pm$ SE	n	%	$\bar{x} \pm$ SE
3+3	10	40	0.67±0.87	9	33	0.77±1.30	9	78	1.44±1.10
4+4	12	50	0.83±1.30	10	90	2.40±1.58	12	83	1.25±0.87
3+4	7	60	0.50±0.84	9	89	2.56±1.33	10	80	1.80±1.32
4+3	10	60	1.10±1.20	10	80	1.60±1.26	5	60	1.60±1.67
Single	11	55[a]	0.73±0.79	10	80	1.60±1.17	9	78	0.89±0.60
Mixed	9	67[a]	1.0±0.87	6	33	0.83±1.33	10	60	1.20±1.32
OP		n=6[b]	1.33±0.52		n=12	1.67±0.65		n=26	1.62±1.02

[a] Although ten pollinations were intended in some cases it was found that more had been carried out

[b] Seed set for open pollinations does not include zeros as capsules were only collected if they had at least one seed

Pollination	Plant								
	1-12			2-1			2-4		
	n	%	$\bar{x} \pm$ SE	n	%	$\bar{x} \pm$ SE	n	%	$\bar{x} \pm$ SE
3+3	11	80	1.70±1.34	12	92	2.58±1.38	12	100	2.83±1.03
4+4	10	90	2.60±1.65	8	89	2.0±1.31	10	100	3.0±0.82
3+4	12	83	2.0±1.28	8	100	2.63±1.19	7	100	2.86±0.90
4+3	10	80	1.90±1.37	12	92	2.58±1.08	10	100	3.0±1.05
Single	10	90	2.4±1.35	10	90	2.20±1.14	10	100	2.80±1.03
Mixed	10	80	2.33±1.73	10	80	2.40±1.35	10	40	1.0±1.49
OP		n=4	2.0±1.15		n=23	3.0±1.0		n=24	2.7±1.20

Pollination	Plant					
	2-5			2-10[a]		
	n	%	$\bar{x} \pm$ SE	n	%	$\bar{x} \pm$ SE
3+3	7	86	1.29±0.95	12	92	1.75±1.22
4+4	10	80	1.50±0.97	–	–	–
3+4	12	82	1.67±1.15	7	88	1.87±1.13
4+3	9	67	1.56±1.42	9	44	0.89±1.17
Single	10	80	2.0±1.33	–	–	–
Mixed	10	50	1.20±1.48	–	–	–
OP		n=8	2.25±0.71		n=24	1.92±1.10

[a] This plant died early in the experiment

Table 3. Two seeds set per capsule side-by-side and diagonally

Plant	Side-by-side	Diagonally	Totals
1-1	11	2	13
1-4	18	2	20
1-6	13	4	17
1-12	9	1	10
2-1	16	3	19
2-4	14	4	18
2-5	16	4	20
2-10	9	1	10

$\chi^2_7 = 2.46$

$\chi^2_1(2:1) = 16.126$

often made it difficult to observe clearly all the features of the embryo sac. The three antipodal nuclei have been reported to be ephemeral, generally disappearing before fertilization (Davis 1966), and were not seen in any of the ovules examined. The polar nuclei occupy a central position and appear to fuse at, or just before, fertilization while the egg apparatus and synergids are found in close association with one another and just below the polar nuclei.

In many of the ovaries examined, the embryo sacs were found to have undergone degenerative changes (Fig. 1a), most noticeably in the endothelium. The cells of the endothelium have narrowed, losing their normal

Table 4. Analysis of variance of seed weight

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Variance ratio
No. seeds/capsule	3	854.0	284.7	10.25
Female parent	6	1778.0	296.3	10.67
Male parent	6	55.4	9.24	0.33
No. seeds × female parent	16	303.2	18.95	0.68
No. seeds × male parent	18	386.6	21.48	0.77
Female parent × male parent	16	428.8	26.8	0.96
No. seeds × female × male	14	234.1	16.72	0.60
Error	302	8387.9	27.77	
Total	381	12428.0		

Table 5. Least square mean seed weights from analysis in Table 4. The overall mean weight of the seeds is 20.44, with a standard error of 0.2922, from 382 observations

Seed no./capsule	1	2	3	4
	22.88	21.68	20.30	18.57
Female parent	1-1	1-4	1-6	1-12
	20.42	17.92	21.36	16.80
	2-1	2-4	2-5	
	22.18	21.29	23.72	

radial expansion, and in some places have broken down entirely, thus no longer separating the embryo sac from the surrounding tissue. Since the commonly presumed functions of an endothelium are to provide protection for, and to channel nutrients to, the embryo sac (Kapil and Tiwari 1978), we believe that there is a strong likelihood that degeneration of the endothelium makes its associated embryo sac inviable. Furthermore, the only distinguishable features in these degenerative embryo sacs were the polar nuclei, the egg apparatus and synergids apparently being absent. We do not know why the endothelium degenerates or whether the disappearance of other components of the embryo is antecedent to or consequent on that degeneration. However, the presence of the polar nuclei makes it highly likely that the degenerative changes occur before fertilization and so are the result of pre-zygotic maternal factors.

Of 79 ovaries examined 59 were found to have an appearance similar to that illustrated in Fig. 1a and only 20 showed no evidence of degenerative changes

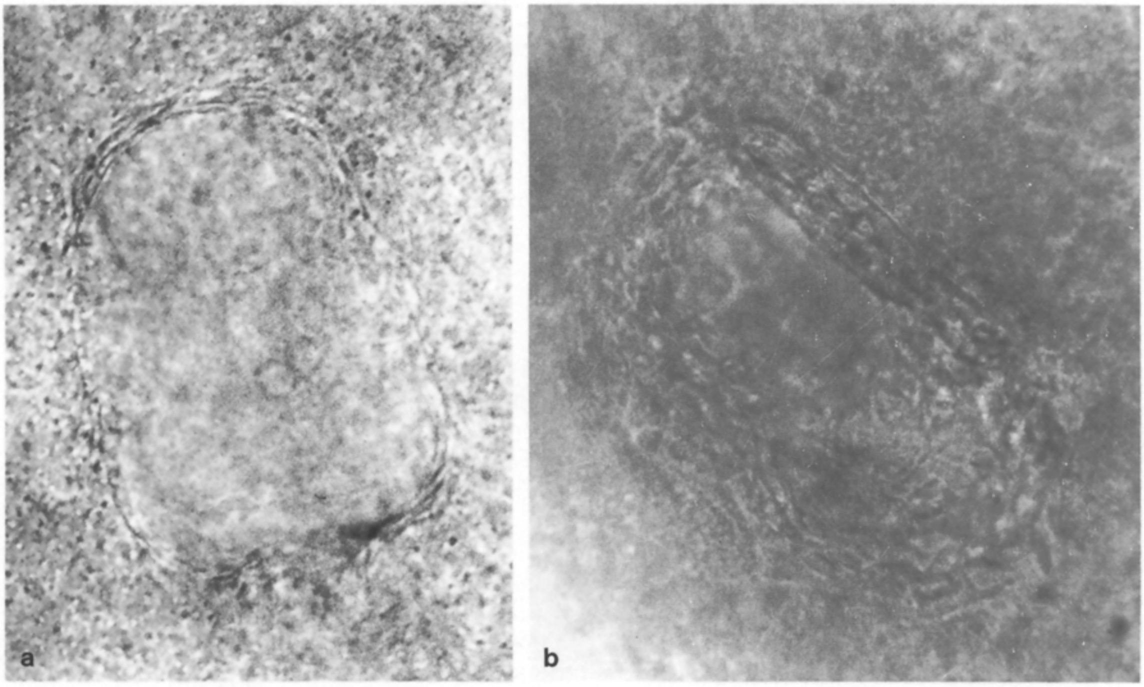

Fig. 1a, b. Borage ovary showing (**a**) degenerative changes as described in text and (**b**) showing normal development

(Fig. 1b). This proportion (23%) of viable embryos is consistent with the proportion of seeds ultimately set. In particular experiments, much higher values have been recorded, but these may have been estimated over the most favourable mid-season flowering conditions. (We have not determined the time course of seed set systematically, but have observed that it is poor when the plants first begin to flower and when senescence starts).

Discussion

The outcome of pollination, shown in Table 1, was analysed using GLIM. Seed set, proportion seed set and arcsine-transformed proportions were all analysed. The outcomes were not different, so analyses are presented on the original (seed number) scale for ease of comparison. First, we considered differences among female parents, then partitioned the results between genotypes (3-3 vs 4-4) and heterogeneity among parents within genotypes (Table 6). In this table, we also show the effects of pollinations: there are no differences between plants used as male parents' at first or second pollination, nor between selfing and crossing. The female effects are highly significant, but because of the heterogeneity among female parents one cannot conclude that the two Diaphorase genotypes differ significantly.

Ganeshaiah and Shaanker (1988) investigated seed set in wind-dispersed pods of a tropical tree, *Dalbergia sissoo*, and found preferential post-fertilization abortion of seeds along the pod, with the highest level at the base, thus depending on the time of fertilization. It is of interest that in the borage flower, while failure of ova occurs before fertilization, there is non-random seed set, the distribution $\begin{smallmatrix}F&F\\S&S\end{smallmatrix}$ of $\begin{smallmatrix}F&S\\F&S\end{smallmatrix}$ being observed more frequently than expected in comparison to $\begin{smallmatrix}F&S\\S&F\end{smallmatrix}$ (F = failure, S = success) on the basis of random failure. The former distribution allows more physical space for development. (All four borage ova are the same distance from the tip of the style, unlike the case in Sissoo).

Ganeshaiah and Shaanker call this phenomenon 'sibling rivalry', a more extreme metaphor than competition for resources. Whatever label is applied, our results show a similar phenomenon. Ganeshaiah and Shaanker tabulate many cases of seed to ovary ratios lower than we have observed in borage (approximately 0.5 over more than 1,000 flowers); one value.of 0.3 for another member of the Boraginaceae (*Cryptantha flava*) has been recorded by Casper and Wiens (1981). In this latter case, ovule abortion was found to be randomly located in the flower, unlike our result, and of course the loss was post-fertilization. Because abortion was very regular, Casper and Wiens speculated that it could be under genetical control. We have shown that there is indeed genetical variation in seed set in borage, where seed set is very variable, but

Table 6. Analyses of variance of seed set

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio
(a) Effect of female parents				
Female parents	7	117.4	16.8	11.6 cf 2.34 (critical value)
3-3 vs 4-4	1	29.2	29.2	
Heterogeneity	6	88.2	14.7	10.2 cf 2.34
Residual	282	407.9	1.45	
Total	289	525.3		
(b) Effect of male and female parents				
Female parents (f)	7	117.4	16.8	11.6
First pollination (p_1)	7	19.7	2.8	2.0
Second pollination (p_2)	7	18.9	2.7	1.9
$f \cdot p_1$	20	23.5	1.2	<1
$f \cdot p_2$	18	16.3	0.9	<1
$p_1 \cdot p_2$	3	2.3	0.8	<1
Residual	227	327.6	1.4	
Total	289	523.5		
(c) Effects of selfing at first and second pollination				
Selfing at first	1	0.66	0.66	5.08
Selfing at second	1	0.13	0.13	
Interaction	1	2.29	2.27	
Residual	286	520.22		
Total	289	523.3		

that environmental factors are also very important. Further, there is genetical variation in seed weight, or at least a natural effect which is independent of the strong influence of number of seeds per capsule. No influence of male parent or seed weight was detected. This analysis has not clarified parental resource allocation in borage, its objective being an understanding of the outbreeding mechanism.

Overall, we conclude that while there are many paths leading to the production of sufficient viable seed for specific survival, that of borage is most unusual.

Acknowledgements. This work was supported by the Australian Research Council. We thank Mrs Karen Goodwins and Mrs Angela Binns for expert technical assistance.

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