New strategies for increasing heterozygosity in crops: *Vicia faba* mating system as a study case

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Summary

Our research assesses the feasibility of using artificial selection on pre-mating floral traits to modify the mating system of faba bean (*Vicia faba*). This analysis considered two synthetic populations, which were derived from different genetic pools and had undergone five years of multiplication. For these populations, we identified floral and inflorescence traits that influence outcrossing per plant and examined the relative importance of these traits in governing yield. Codominant isozyme loci and the mixed-mating model were used to estimate the multilocus female outcrossing rate. A maternal half-sib design was used to evaluate the additive genetic component of floral and inflorescence traits, yield and yield components. Multiple regression was used to assess the effects of floral and inflorescence traits on outcrossing and yield and components of yield. The two populations exhibited mixing mating. Self-fertilization appears to result from the action of pollinating bees, so that its incidence could be modified by selection on floral and inflorescence traits that affect pollination. Floral and inflorescence traits affected individual differences in outcrossing unequally, with most variation being associated with the numbers of displayed flowers and inflorescences. Variation among plants in reward traits and in shape, although statistically significant, had limited and inconsistent influences on individual differences in outcrossing. Yield and its components varied strongly with aspects of floral display and, to a lesser extent, floral design, except for seed weight. Overall, our results imply that both outcrossing and yield could be enhanced by selection for plants that produce more inflorescences, each with relatively few flowers.

Introduction

Plant breeding for sustainable agriculture selects cultivars that fit the environment, rather than altering the environment to fit the cultivars (Ceccarelli et al., 1992). Such breeding programs typically seek cultivars with enhanced resistance or tolerance to pests and pathogens (Sleeper et al., 1991). However, a crop's biotic environment also includes beneficial organisms, which can be employed to increase yield and stability. For animal-pollinated, partially allogamous crops, pollinators are particularly important in this context, because their activity determines both the incidence of ovule fertilization and its genetic character (self- versus cross-fertilization: Free, 1993; Bond & Kirby, 2001). Therefore, the efficiency of plant breeding programs for integrated agriculture may be improved by the analysis and exploitation of plant-pollinator interaction (de Oliveira, 1997).

The functional relations of plants and their pollen vectors are particularly important in the development of synthetic varieties. Such varieties are created by hybridizing several selected genotypes, which increases heterosis and thereby enhances yield and stability (Bond, 1982). To maintain this heterosis, synthetic varieties are maintained by persistent outcrossing under

open pollination, which requires the service of pollen vectors. Consequently, the development and maintenance of synthetic varieties should be assisted by including plant traits that promote cross-pollination as targets of the breeding program. For animal-pollinated plants, the incidence of cross-pollination depends on aspects of both floral design (flower form, size, colour, nectar production, etc.) and floral display (number of open flowers, inflorescence architecture), which influence pollinator attraction, pollen removal and pollen deposition (Harder & Barrett, 1996). Therefore, an understanding of how these traits influence pollen vectors and plant mating should allow plant breeders to select cultivars that outcross consistently. This knowledge-based approach should allow for more efficient development of high-yielding synthetic varieties, and has been employed profitably to improve yield of wind-pollinated cereals (Virmani, 1996; Ghani et al., 2003). However, floral and inflorescence traits that promote outcrossing have received less attention in the development of animal-pollinated crops.

Development of synthetic varieties has been promoted as an effective strategy for increasing yield and stability of *Vicia faba* L. (Bond, 1989; Stelling et al., 1994; Link et al., 1994; Duc, 1997; Gasim et al., 2004), a partially outcrossing, bee-pollinated crop, which shows significant and valuable heterosis (Stoddard & Bond, 1987). Studies of the influences of floral traits on outcrossing by faba bean have focused on a few floral characters. Knudsen & Poulsen (1981) reported that cross-pollination declined 50% after the introduction of a closed-flower gene. Currie et al. (1990) reported that inter-plot outcrossing correlated with the frequency with which bees moved between cultivars, and that bees tended not to visit smaller, spindly cultivars. Flower structure seems to be a major influence on self-fertility, as a relatively short style, which bends at ~90° enhances self-pollination in inbred lines (Kambal et al., 1976). Finally, Carre et al. (1993) found that bumble bees (*Bombus terrestris*) visited inbred lines primarily to collect pollen, and that differences in outcrossing rate among lines were related to differences in self-incompatibility.

Our research seeks to identify floral and inflorescence traits (floral morphology, structure, rewards and advertisement, inflorescence distribution in time and space) that affect outcrossing and yield, which will be the target of future selection in the development of new faba bean varieties that optimise the plant-pollinator interaction. Therefore, the main objectives of this study were: (1) to examine quantitative variation in floral traits; and (2) to describe statistically the relation of outcrossing rate and yield to the additive genetic component of floral traits, as expressed by floral-trait means for half-sib families. Information revealed by this research will also help research partners world-wide to produce heterotic varieties by building populations within which heterozygosity is maintained by floral behaviour. Even if heterozygosity varies from year to year, this means of exploiting it is preferable to the use of conventional hybrids from the sustainable standpoint.

Material and methods

Plant material

Seven lines, belonging to the four botanical groups of faba bean were selected from the world germplasm collection of the IAS-CIFA (Cordoba, Spain) to develop two experimental synthetic populations, syn-4 and syn-5. Experimental populations were originally constructed by mixing identical numbers of seeds from the parental lines (six lines for syn-4, and all seven lines for syn-5) as described by Maalouf et al. (1999). These populations have since been multiplied for five years in open-pollination conditions, with the indigenous, solitary bee *Eucera numida* Lep. serving as the principal pollinator (M.J. Suso, personal observation). These populations were not subject to artificial selection before being used for this experiment. In addition, production plots were isolated from each other by 3-m wide plots of rapeseed to prevent gene flow among the synthetic varieties.

Experimental design

Field experiments were carried out at Cordoba, southern Spain during two seasons following cultural practices described by Maalouf et al. (2002). Plants were grown in rows with 70 cm between rows and 10 cm between adjacent plants in a row. We estimated the female outcrossing rate (t: proportion of seeds that were outcrossed) for fifty maternal plants based on seeds collected during the 2001/2002 growing season from both synthetic populations (details below). To characterise the additive genetic component of these plants' floral and yield phenotypes, we measured average traits for 14 offspring per maternal parent during the 2002/2003 growing season. These maternal half-sib families were grown in a completely randomised design with two replicates.

Estimation of the female outcrossing rate

We estimated the female outcrossing rate of the 50 plants collected during 2001/2002 from each synthetic population based on 15 seeds/plant. Suso et al. (1993) describes the protocols for enzyme extraction, PAG electrophoresis and the stain recipes. Seven polymorphic loci were scored: *6Pgd*, *Skd*, *Est*, *Sod-1*, *Sod-2*, *Sod-3*, and *Idh*. Female outcrossing rates were estimated by maximum likelihood for the synthetic populations as a whole and for individual plants using Ritland's (1990, 2002) multilocus estimation program, MLTR. Although MLTR sometimes fails to converge on an estimate when applied to individual plants, this problem did not arise in our experiment. For the population as a whole, we estimated three mating parameters: the multilocus female outcrossing rate (t_m); the single-locus female outcrossing rate (t_s), which is an average across loci; and the parent's fixation index. Estimation of the multilocus outcrossing rate analysis used the Newton–Raphson method for parameter estimation and standard errors for were calculated from 1000 bootstraps, with the progeny array as the unit of resampling. The expected equilibrium inbreeding coefficient was calculated from the multilocus outcrossing rate, according to $F_e = (1 - t_m)/(1 + t_m)$.

Measurement of floral and inflorescence traits

We classified reproductive traits into three categories: (1) phenology, (2) design and (3) display. Phenology was recorded for 750 plants in each synthetic population (7 plants per maternal family and two replications). We summarized individual phenologies based on first flowering date (FO), last flowering date (FF) and flowering duration (CICLO = FF − FO).

The floral design traits chosen for analysis included nectar volume and concentration, and a variety of simple and composite measures of the standard and keel petals and the ovary. We measured flowers for six plants from each maternal family in one replicate of each synthetic population. For each plant, we measured two flowers from the fifth, sixth, or seventh node of each plant. We specifically chose "open flowers", as characterised by Osborne et al. (1997), because bees preferentially visit flowers in this stage, presumably because they are the easiest to manipulate and most attractive and rewarding. The mean measurement of the two flowers was used as a plant's phenotypic value. To measure nectar volume and concentration we extracted nectar from flowers during early morning with

calibrated, disposable microcapillary tubes. The length of the nectar column in the tube was measured and the volume of nectar calculated. Sugar concentration (% sucrose per mass of nectar) of this nectar was measured with a pocket refractrometer which had been adjusted to handle small volumes (Bellingham and Stanley Ltd.).To measure flower size and shape, we used a digital scanner to record images of individual flowers and analyzed these images with the UTHSCSA *Image Tool* program (http://www.uthsca.edu/dig/itdesc.html). For each flower image, we measured the following characteristics in cm (Figure 1): maximum length of the standard petal (LTC), standard width (ANE), standard perimeter (PERE), length of the keel petal (LQ), keel width (ANQ), keel perimeter (PERQ), keel opening (APQ), floral-tube length (LTB), style length (LE), ovary (LO) length, ovule number (OVUL), and the angle between the style and ovary (ANGU). We also calculated two ratio variables: LTC/ANE and LTC/LQ. Following Cresswell (1998), we classified design traits into functional categories: advertisement (e.g., standard dimensions), female sexual dimension (e.g., ovule number), reward (e.g., nectar volume and concentration), and fit to pollinator (e.g., keel dimensions and floral-tube length).

We measured floral display traits for 700 plants in each synthetic population (7 plants per maternal family, 50 maternal families and two replicates). Once each week for the four weeks after a plant's first flower opened, we counted the number of open flowers (NFA) and the number of inflorescences with open flowers (NNFA). We estimated a plant's total flower production (NTFA) and total inflorescence production (NTNF) as the sums of NFA and NNFA, respectively, during the same period.

Once plants set seeds, we weighed the total yield per plant (RP, in grams) and partitioned it into the total number of seeds (NGP) and pods (NVP), the number of seeds per pod (NGV), and the average mass of 100 seeds (P100S). Yield traits were measured for 750 plants in each synthetic population (seven plants per maternal family and two replications).

Statistical methods

We used two-factor analyses of variance (Neter et al., 1996) to identify significant variation among maternal families (random effect) and replicates for phenology, floral display and design, and yield and its components. This analysis was conducted separately for each synthetic population. All analyses were performed

Figure 1. Floral and inflorescence traits measured.

with the GLM (General Linear Model) procedure in SAS.

We used multiple regression analysis (Neter et al., 1996) to investigate whether outcrossing, yield and yield components varied significantly with floral and inflorescence traits, to describe the nature of this relation and to determine which traits could account for the large proportion of the variation among plants. These analyses considered two perspectives. The analysis of maternal outcrossing rate is retrospective in that it uses the average traits of each maternal plant's offspring as a measure of the maternal phenotype. In contrast, analyses of yield and yield components consider the average performance of each maternal plant's offspring. Family means of floral and inflorescence traits were averaged over six plants, whereas phenology and yield traits were averaged over 14 plants. A stepwise procedure was used to select independent variables that together influenced aspects of plant performance significantly. Outcrossing rate was arcsine square-root transformed and other variables were suitably transformed to conform with the analysis assumptions. We report standardized regression coefficients, which relate the magnitude of change in a dependent variable associated with a change in an independent variable, measured in standard deviations. Multiple regression analysis was conducted with Statistica v.6 (StatSoft).

Less than 70% of sampled plants produced nectar and the remainder were nectarless, so we derived two groups of regression models for each synthetic population: one model considered all plants, whereas the second model considered only nectar-producing plants. The analysis of all plants included a binary independent variable representing the presence of nectar: sugar concentration was not included in this analysis.

Results

Population outcrossing rate and genetic structure

Both synthetic populations retain evidence of their history of inbreeding. Maternal parents in syn-5 had an inbreeding coefficient (F) of 0.23 (95% confidence interval, 0.07–0.39). This incidence of inbreeding approximately matches that expected at the inbreeding equilibrium with no inbreeding depression $F_e = 0.32$. In contrast, the syn-4 population had a much lower inbreeding coefficient (F) of 0.09 (95% confidence limits, −0.06 and 0.24). This incidence of inbreeding is less than the equilibrium inbreeding coefficient (0.30), indicating an excess of heterozygous plants in the syn-4 population, which may have resulted from

heterozygote advantage due to heterotic selection (Clegg, 1980; Rüter et al., 2000).

During our experiment, both synthetic populations exhibited mixed mating, with about 50% of progeny resulting from outcrossing, on average ($t_m \pm$ S.E., 95% confidence interval: syn-4 population, 0.51 ± 0.08, 0.36–0.66; syn-5 population, 0.48 ± 0.04, 0.40–0.56). Outcrossing varied extensively among families in both populations (Figure 2). Although some families were completely selfed and others were completely out-

crossed, most families comprised a mixture of selfed and outcrossed progeny. Multilocus outcrossing rates did not differ significantly from the mean of the single-locus outcrossing estimates in either population, supporting earlier findings that selfing is the principal form of inbreeding in faba beans (Suso et al., 2001). Although some families in both populations did not produce nectar, the outcrossing rates of nectarless and nectar-producing families did not differ significantly ($P > 0.5$ in both populations).
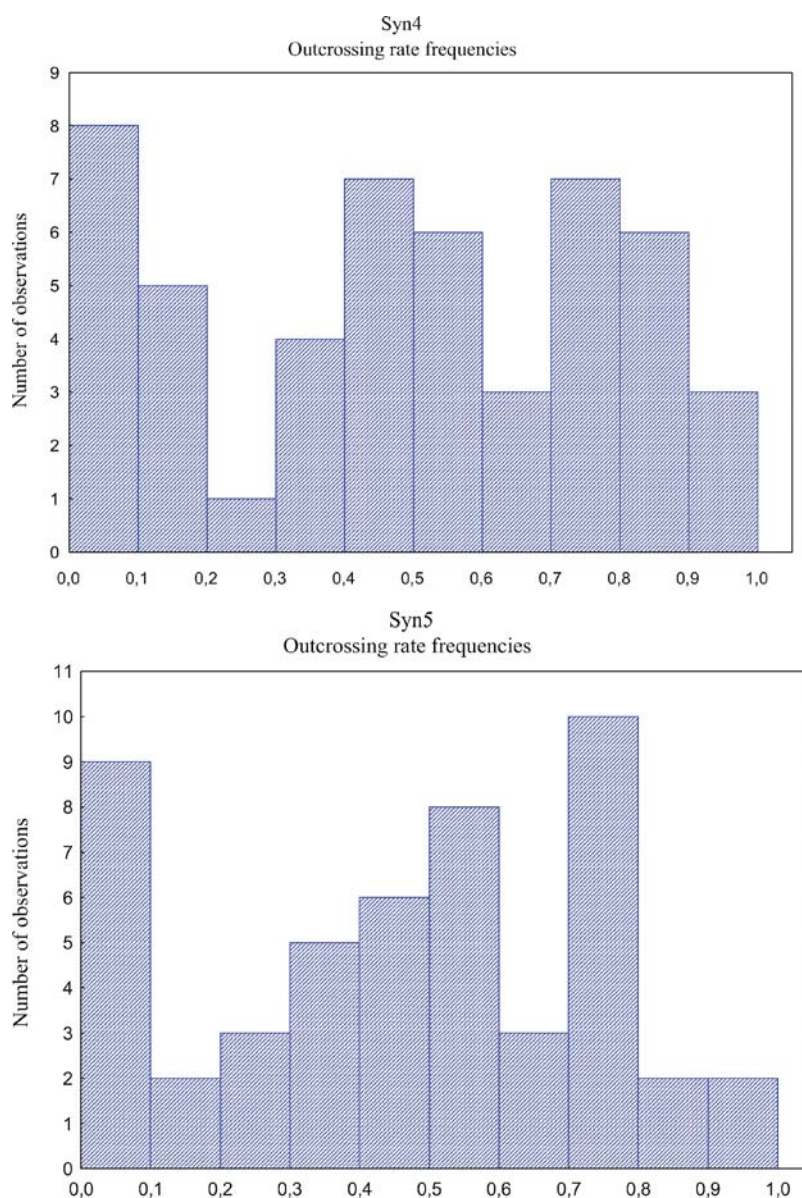

Figure 2. Frequency distribution of female outcrossing rates among the 50 maternal plants from the Syn4 and Syn5 populations during the 2001/2002 growing season.

Patterns of variation in floral and inflorescence traits

Overall, the two synthetic populations had similar floral characteristics (Table 1). The greatest difference resulted from the threefold greater nectar production by the syn-4 population.

Floral traits differed strongly in the amount of variation within populations, as measured by the coefficient of variation (Table 1). Nectar volume varied most, primarily because of qualitative differences among plants, as only 69% and 54% of plants produced nectar in the syn-4 and syn-5 populations, respectively. The least variable floral traits involved two female sexual dimensions, ovary (LO) and style length (LE), and floral-tube length (LTB). Plants differed broadly in floral display, particularly the numbers of open inflorescences and flowers during the first week of flowering. Much of this variation resulted from differences in flowering phenology, as the CV for first flowering date (FO) was about 20% in both populations, whereas that for last flowering date (FF) was only about 5%. Yield (RP) varied widely among plants in both populations, with seeds per plant (NGP) varying most extensively, especially in the syn-5 population. This result arose primarily from differences among plants in pod production (NGV) and average seed mass (P100S), as the number of seeds per pod (NGV) varied relatively little.

The extent of variation among maternal families differed among traits (results not shown). All phenological traits varied significant among maternal families, with flowering duration having the largest variance component. Aspects of floral display, exhibited more complicated variation than other sets of traits. All display traits varied significantly among families; however, the pattern of variation differed among replicates for the numbers of open flowers during the second (NFA2) and third weeks (NFA3) of flowering in both populations, and the numbers of open inflorescences during the third (NNFA3) and fourth (NNFA4) weeks in the syn-5 population. Other than nectar traits, all aspects of floral design varied significantly among maternal families, with standard perimeter (PERE) in syn-4 and ovule number (OVUL) in syn-5 having the largest variance components. Yield and its components also varied significantly among maternal families, except for the total number of seeds per plant (NGP) in both populations and pod production per plant (NVP) in the syn-4 population.

Effects of floral and inflorescence traits on outcrossing

Outcrossing by maternal plants varied more strongly with the mean floral traits of their offspring in the syn-4 population than in the syn-5 population (Table 2). For the syn-5 population, only standard length/width (LTC/ANE) affected maternal outcrossing rate significantly, with families with relatively short, broad standard petals being more outcrossed. In contrast, four display traits accounted for 42% of the variation in maternal outcrossing among families in the syn-4 population. All four traits had effects of similar magnitude, based on their standardized partial regression coefficients, but the directions of these effects differed. In particular, the offspring of highly outcrossing maternal plants had more flowers open on fewer inflorescences during the second week and they produced more inflorescences, but fewer flowers overall. These results reveal contrasting effects of flower and inflorescence production and presentation.

Floral traits explained much more of the variation in outcrossing for nectar-producing plants than for the entire sample for both synthetic populations (Table 2). More than 80% of the variation in outcrossing could be attributed to variation in floral display and design and phenology. Based on the absolute values of the standardized partial regression coefficients, aspects of floral display affect outcrossing rates most strongly for both populations, especially the numbers of open flowers (negative effect) and inflorescences (positive effect) during the first week of flowering and the number of flowers during the third week of flowering. The timing of flowering (first and last flowering date) did not affect outcrossing rates significantly; however, the mothers of families with long flowering periods had higher outcrossing rates in the syn-4 population. For both populations, outcrossing declined with increasing flower depth (LTB) and nectar production (NECT in syn-5 and AZU in syn-4). Outcrossing also varied in both populations with the ratio of standard length to width (LTC/ANE); however, the effect differed between the populations, being positive in syn-4 and negative in syn-5. Several aspects of floral design also had unique effects on outcrossing in the two populations. In syn-5, outcrossing rate varied negatively with the ratio of the lengths of the standard and keel petals (TLC/LQ). In syn-4, outcrossing rate varied negatively with the perimeter of the standard petal and positively with the length of the keel petal and ovule number.

Table 1. Basic statistics for floral traits in the two synthetic populations

Trait*	Syn-4				Syn-5			
	Mean ± S.D.	CV	Min	Max	Mean ± S.D.	CV	Min	Max
Floral display								
NNFA1	3.60 ± 0.84	23	0.8	5.4	3.68 ± 1.02	28	0.8	6.2
NNFA2	5.36 ± 0.78	15	2.4	6.7	5.44 ± 0.88	16	3.0	7.8
NNFA3	4.13 ± 0.50	12	3.1	5.1	3.67 ± 0.58	16	2.9	6.4
NNFA4	3.10 ± 0.29	9	2.5	4.2	3.09 ± 0.83	27	2.2	7.6
NTNF	16.2 ± 1.67	10	10.6	19.4	15.90 ± 20	12	11.8	21.4
NFA1	10.9 ± 3.30	30	2.5	19.0	12.26 ± 4.3	35	2.3	22.2
NFA2	18.3 ± 3.34	18	7.8	25.2	18.52 ± 3.3	18	9.0	27.9
NFA3	14.1 ± 2.44	17	9.6	19.4	12.29 ± 2.3	19	7.4	19.0
NFA4	9.47 ± 1.75	18	6.5	16.7	8.07 ± 1.91	24	4.6	15.4
NTFA	52.8 ± 7.50	14	33.5	68.4	51.27 ± 7.69	15	31.4	72.0
Phenology (days)								
FO	13.1 ± 3.41	26	4.4	22.4	14.75 ± 2.99	20	7.9	22.8
FF	54.9 ± 3.77	6	49.4	65.5	54.27 ± 2.53	5	50.1	61.3
CICLO	41.4 ± 5.19	12	32.1	53.7	39.53 ± 4.08	10	30.4	46.9
Advertisement (cm)								
LTC	3.16 ± 0.16	5	2.8	3.6	3.12 ± 0.12	4	2.9	3.4
ANE	1.32 ± 0.15	11	1.0	1.8	1.24 ± 0.09	7	1.0	1.5
PERE	7.59 ± 0.35	5	6.7	8.3	7.43 ± 0.29	4	6.9	8.0
LTC/ANE	2.42 ± 0.24	10	1.9	3.1	2.53 ± 0.15	6	2.2	3.0
Female dimension								
OVUL	3.62 ± 0.37	10	2.8	4.7	3.68 ± 0.41	11	2.5	4.8
LO (cm)	1.70 ± 0.06	4	1.6	1.9	1.71 ± 0.07	4	1.6	1.9
LE (cm)	0.44 ± 0.02	3	0.4	0.5	0.44 ± 0.02	3	0.4	0.5
ANGU	64.46 ± 4.75	7	54.5	76.3	67.27 ± 3.85	6	59.5	77.5
Fit to pollinator (cm)								
LTB	1.33 ± 0.05	4	1.2	1.4	1.33 ± 0.07	5	1.2	1.5
LQ	1.59 ± 0.06	4	1.5	1.7	1.62 ± 0.06	4	1.5	1.8
ANQ	0.68 ± 0.03	5	0.6	0.7	0.67 ± 0.03	4	0.6	0.7
PERQ	3.39 ± 0.15	4	3.0	3.7	3.41 ± 0.14	4	3.2	3.9
APQ	0.68 ± 0.04	6	0.6	0.8	0.69 ± 0.04	5	0.6	0.78
LTC/LQ	1.97 ± 0.1	5	1.8	2.3	1.93 ± 0.07	4	1.8	2.1
Reward								
NECT (μl)	0.11 ± 0.15	129	0.0	0.8	0.03 ± 0.05	160	0	0.3
AZU (g)	0.67 ± 0.12	18	0.4	0.9	0.70 ± 0.12	17	0.5	1.0
Yield components								
RP (g)	33.00 ± 8.66	26	17.1	60.3	29.86 ± 9.37	31	14.5	64.8
NGP	53.90 ± 11.9	22	32.0	87.6	54.67 ± 14.92	27	28.4	95.8
NVP	18.90 ± 3.66	19	11.5	27.6	18.95 ± 5.2	27	9.2	34.3
NGV	2.82 ± 0.27	9	2.1	3.5	2.90 ± 0.24	8	2.4	3.4
P100S (g)	60.5 ± 10.1	16	42.3	99.6	53.23 ± 7.82	14	31.3	72.4

*Floral display: Numbers of inflorescences (NNFA1, NNFA2, NNFA3, NNFA4) and open flowers (NFA1, NFA2, NFA3, NFA4) during first, second, third and fourth weeks of flowering, and total inflorescence (NTNF) and flower production (NTFA). Phenology: day of first (FO) and last flowering in the population (FF), and flowering duration (CICLO). Advertisement: standard-petal length (LTC), width (ANE), perimeter (PERE) and length:width ratio (LTC/ANE). Female sexual dimension: ovule number (OVUL); lengths of the ovary (LO) and style (LE); angle between the style and ovary (ANGU). Fit to pollinator: corolla-tube length of (LTB); keel-petal length (LQ), width (ANQ) and keel perimeter (PERQ); length of the keel opening (APQ); ratio of the length of the standard and keel petals (LTC/LQ). Reward: nectar volume (NECT) and concentration (AZU).Yield: total seed mass (RP); total seed number (NGP); total pod number (NVP); the number of seeds per pod (NGV); average mass of 100 seeds (P100S).

Table 2. Summary of regressions of outcrossing by maternal plants on average characteristics of floral display, floral design and phenology among their offspring

Population	Group	R^2	df_e	Independent variable	Standardized regression Coefficient ± S.E.	t
Syn-5	All plants	0.19	45	LTC/ANE	−0.40 ± 0.13	2.15**
	Nectar-producing	0.94	11	NNFA1	1.90 ± 0.38	5.00***
				NFA1	−1.51 ± 0.31	4.85***
				NFA3	1.44 ± 0.28	5.12***
				LTC/ANE	−0.55 ± 0.09	5.73***
				LTB	−0.84 ± 0.13	6.43***
				LTC/LQ	−0.59 ± 0.11	5.06***
				NECT	−0.26 ± 0.08	2.99*
Syn-4	All plants	0.42	40	NNFA2	−1.44 ± 0.46	3.14**
				NTNF	1.11 ± 0.46	2.38*
				NFA2	1.12 ± 0.40	2.79**
				NTFA	−1.10 ± 0.37	2.98**
	Nectar-producing	0.81	17	NNFA1	1.19 ± 0.39	3.02**
				NFA1	−1.39 ± 0.41	3.35**
				NFA3	−0.58 ± 0.16	3.48**
				CICLO	0.88 ± 0.32	2.69*
				PERE	−0.84 ± 0.35	2.39*
				LTC/ANE	0.77 ± 0.32	2.38*
				OVUL	0.43 ± 0.15	2.75*
				LTB	−0.31 ± 0.14	2.19*
				LQ	0.67 ± 0.24	2.74*
				AZU	−0.35 ± 0.15	2.27*

Note. Only significant independent variables are shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
Floral display: open flowers (NFA1, NFA2, NFA3) and inflorescences (NNFA1, NNFA2) at first, second and third week of flowering; total open flowers NTFA and inflorescences NTNF. Phenology: duration of flowering (CICLO). Floral design: (A) Advertising: standard length (LTC), width (ANE) and perimeter (PERE); (B) Fit to pollinator: tube length (LTB), keel length (LQ); (C) Female sexual dimension: (OVUL); (D) Reward: nectar volume (NECT) and sugar concentration (AZU).

Effects of floral and inflorescence traits on yield and yield components

Yield per plant (=total seed mass) by the offspring generation varied among families primarily with aspects of floral display (Table 3). In the syn-5 population, plants that produced more inflorescences (NTNF) tended to yield more, regardless of whether the analysis considered all plants, or only nectar-producing plants. The analysis for the total sample from this population also detected a significant negative influence of floral display during the first week of flowering (NFA1) and a positive effect of floral-tube length (LTB). For the syn-4 population, the mass of seeds produced per plant varied significantly, and positively, only with the number of inflorescences open during the fourth week of flowering (NNFA4).

The influences on the four yield components that we considered, seed mass, seeds per plant, pods per plant and seeds per pod, varied extensively among components and differed between the two synthetic populations (Table 4). Average seed mass did not vary significantly with any of the traits that we measured (results not shown). In contrast, aspects of floral design and display explained at least half of the variation in the remaining yield components, except for the analyses of seeds per plant for the entire samples for both populations ($R^2 > 0.3$ in both cases), and the analysis of seeds

Table 3. Summary of regressions of the total mass of seeds (yield) produced by families on their average characteristics of floral display, floral design and phenology

Population	Group	R^2	df_e	Independent variable	Standardized regression Coefficient ± S.E.	t
Syn-5	All plants	0.39	42	NTNF	0.94 ± 0.21	4.31**
				NFA1	−0.57 ± 0.22	2.56*
				LTB	0.28 ± 0.13	2.03*
	Nectar-producing	0.46	17	NTNF	0.45 ± 0.28	2.15*
Syn-4	All plants	0.39	42	NNFA4	0.54 ± 0.15	3.49**
	Nectar-producing	0.50	27	NNFA4	0.38 ± 0.14	2.7*

Note. Only significant independent variables are shown.
* $P < 0.05$, ** $P < 0.001$.
Floral display: open flowers (NFA1) at the first week, open inflorescences (NNFA4) at the fourth week of flowering; total open inflorescences NTNF. Floral design: tube length (LTB).

per pod for the entire sample from the syn-5 population ($P > 0.05$). In general, features of floral display were the predominant influences on seeds per plant and pods per plant for the syn-5 population, whereas in the syn-4 population aspects of floral design were equally important. Phenological traits had relatively little influence on yield components, although the date of first flowering positively affected seeds per pod and seeds per plant for nectar-producing plants in the syn-4 population, and the date of last flowering negatively influenced pod production in the total sample for the syn-5 population.

Aspects of floral display that influenced yield components typically included either total flower (NTFA) or inflorescence production (NTNF), or display size during the two last weeks of flowering (NFA3, NFA4 and NNFA3, NNFA4, respectively), rather than during the early flowering period (Table 4). These display characteristics generally influenced yield components positively. The most consistent exception resulted from negative effects of inflorescence production during the fourth week of flowering and total flower production on the number of pods per plant.

Features of floral design affected yield components primarily for nectar-producing plants, rather than the entire samples (Table 4). The most consistent exception to this involved ovule number, which negatively affected seeds per plant and positively affected seeds per pod in the syn-4 population, and negatively influenced pods per plant in the syn-5 population. In addition, long, slender flowers, as measured by floral-tube length (LTB) and the ratio of standard length to width (LTC/ANE) tended to produce more pods per plant in

the entire sample for the syn-5 population. The numbers of seeds produced by nectar-producing plants from the syn-4 population also varied positively with keel width (LQ) and negatively with style length (LE), the angle between the style and ovary (ANGU) and nectar volume (NECT). For nectar-producing plants from the syn-5 population, plants with relatively long standards compared to their keels (LTC/LQ) tended to produce fewer seeds. Pod production by nectar-producing plants from the syn-4 population varied positively with standard length (LTC) and negatively with the ratio of standard and keel lengths (LTC/LQ) and style length (LE). Finally, the number of seeds per pod for nectar-producing plants varied positively with ovule number (OVUL) and keel width (ANQ) for the syn-4 population, whereas for the syn-5 population seeds per pod varied positively with standard length (LTC) and negatively with standard width (ANE), the ratio of standard length to standard width (LTC/ANE) and the perimeter of the standard petal (PERE).

Discussion

Food legumes, in general, and *Vicia faba*, in particular, are important protein sources for animal and human consumption (Baum et al., 1997) and need to be integrated in a sustainable agriculture (Byerlee & White, 2000). However, this integration requires the development of improved varieties, because sustainable agricultural systems have higher demands on yield stability than does conventional agriculture (Smithson & Lenné, 1996). The development of synthetic varieties has been

Table 4. Summary of regressions of average yield components by families on their average characteristics of floral display, floral design, and phenology

Dependent variable	Population	Group	R^2	df_e	Independent variable	Standardized regression Coefficient ± S.E.	t
Seeds/plant	Syn-5	All plants	0.42	41	NTNF	0.60 ± 0.21	2.19*
					NFA4	-0.38 ± 0.16	2.37*
		Nectar-producing	0.61	17	NFA4	-0.49 ± 0.17	2.82*
					LTC/LQ	-0.41 ± 0.18	2.24*
	Syn-4	All plants	0.31	42	NNFA4	0.35 ± 0.14	2.44*
					OVUL	-0.35 ± 0.13	2.55*
		Nectar-producing	0.52	26	NNFA4	0.68 ± 0.21	3.15**
					FO	0.45 ± 0.16	2.84**
					LE	-0.50 ± 0.17	2.87**
					ANGU	-0.37 ± 0.15	2.43*
					LQ	0.54 ± 0.16	3.34**
					NECT	-0.32 ± 0.16	2.08*
Pods/plant	Syn-5	All plants	0.54	39	NNFA3	0.64 ± 0.21	2.94**
					NTFA	-0.98 ± 0.36	2.70*
					FF	-0.41 ± 0.19	2.15*
					OVUL	-0.37 ± 0.12	3.07**
		Nectar-producing	0.75	14	NNFA4	-0.63 ± 0.22	2.77*
					NTNF	1.32 ± 0.58	2.25*
					NFA3	0.80 ± 0.29	2.75*
	Syn-4	All plants	0.51	34	NNFA2	0.98 ± 0.41	2.36*
					NNFA4	0.51 ± 0.21	2.40*
					LTC/ANE	0.43 ± 0.14	2.95**
					LTB	0.33 ± 0.16	2.02*
		Nectar-producing	0.81	19	NNFA2	0.88 ± 0.26	3.38**
					NNFA4	0.50 ± 0.13	3.85**
					NTFA	-0.79 ± 0.26	2.95**
					LTC	2.06 ± 0.56	3.64**
					LE	-0.57 ± 0.14	3.88**
					LTC/LQ	-1.60 ± 0.49	3.26**
Seeds/pod	Syn-5	Nectar-producing	0.53	15	LTC	1.40 ± 0.54	2.55*
					ANE	-2.81 ± 1.09	2.58*
					LTC/ANE	-2.25 ± 0.95	2.36*
	Syn-4	All plants	0.77	42	NFA3	-0.58 ± 0.14	4.16***
					NFA4	0.26 ± 0.08	2.93**
					OVUL	0.82 ± 0.07	10.46***
		Nectar-producing	0.61	23	NFA3	-0.67 ± 0.24	2.74*
					NFA4	0.39 ± 0.16	2.44*
					FO	0.33 ± 0.14	2.25*
					PERE	-0.79 ± 0.32	2.43*
					OVUL	0.41 ± 0.15	2.70*
					ANQ	0.65 ± 0.23	2.79*

Note. Only significant independent variables are shown.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
Floral display: open flowers (NFA3, NFA4) and inflorescences (NNFA2, NNFA3, NNFA4) at second, third and fourth week of flowering; total open flowers NTFA and inflorescences NTNF. Phenology: first (FO) and last (FF) flowering date. Floral design: (A) Advertising: standard length (LTC), width (ANE), perimeter (PERE); (B) Fit to pollinator: tube length (LTB), keel length (LQ) and width (ANQ); (C) Female sexual dimension: (OVUL), style length (LE) and style-ovary angle (ANGU); (D) Reward: nectar volume (NECT).

promoted as an effective strategy for increasing yield and stability in *Vicia faba* L. (Stelling et al., 1994; Link et al., 1994; Duc, 1997; Gasim et al., 2004) as a consequence of elevated heterozygosity, and the integration of pollinators.

Enhancement of outcrossing during the development of synthetic varieties, although complicated by the partial allogamy of faba bean, is necessary because the outcrossing rate is a key component of variety performance (Ebmeyer & Stelling, 1994). Our estimates indicate that faba bean cultivars have a mixing mating system ($t \approx 50\%$: Figure 2), with substantial self- and cross-fertilization (also see Suso & Moreno, 1999; Suso et al., 2001). Furthermore, the similarity of single- and multilocus outcrossing rates indicate that selfing is the most important form of inbreeding in faba beans (also see Suso et al., 2001). Consequently, there is considerable scope for selective plant breeding to increase outcrossing by faba bean through the development of synthetic varieties.

Despite the potential for increased heterozygosity to enhance yield potential and stability, this goal has been elusive in faba bean breeding, because this species is not fully responsive to the exploitation of heterosis by usual methods, such as increased hybridisation rate, cytoplasmic and genetic male sterility, hand emasculation, self-incompatibility, etc. (Berthaut et al., 1991; Le Guen et al., 1991; Link et al., 1997). Some researchers have advocated selection of pre-mating floral traits to generate open varieties with high heterozygosis (Ceccarelli, 1978; Ghani et al., 2003), which also avoids the dangers of genetic uniformity associated with the development of hybrid varieties. However, this approach requires an understanding of the relation of outcrossing to floral traits, which is mediated by the action of pollinators, and its genetics. A number of breeding programs have been described for using pre-mating floral characteristics to produce superior cultivars (Virmani & Athwal, 1973; Geiger et al., 1994; Virmani, 1996). In addition, Metz et al. (1993) reported significant differences among genotypes in cross-fertilization, although they found few genotypes with high cross-pollination. Metz et al. suggested that a three-component variety required outcrossing rates of at least 75%. Plants exceeding this frequency of outcrossing were found in our experiment (Figure 2).

Effects of floral and inflorescence traits on outcrossing

Within and between species, flowers of regularly selfing forms have smaller petals, sepals, anthers, and styles, than outcrossing forms, but ovary length does not differ (e.g., Schoen, 1982; Motten & Antonovics, 1992; Georgiady & Lord, 2002). In such cases, selfing occurs predominately within, rather than between, flowers and typically does not require the action of pollinators. Consequently, the incidence of self-fertilization by plants that typically self-pollinate autonomously should be unrelated to aspects of floral display, although this relation seems not to have been examined. In contrast, in outcrossing species with mixed mating systems, self-pollination often results from interaction with pollinators, particularly the tendency of pollinators to visit several flowers on the same plant, which causes geitonogamy (Schoen & Lloyd, 1992; Barrett et al., 1994; Leclerc-Potvin & Ritland, 1994; Eckert, 2000; Karron et al., 2004). Our regression analysis detected significant effects of floral design and display on the mating system (Table 2), which are most consistent with a roles of pollinator activity in both self- and cross-pollination.

Several aspects of floral design varied significantly with maternal outcrossing rate within the two synthetic populations that we studied, particularly among nectar-producing plants (Table 2). In general, the offspring of predominately outcrossing plants had relatively short standard petals compared to their keel petals (LTC/ANE) and short floral tubes (LTB). Plants in largely outcrossed families also produced smaller volumes of less-concentrated nectar (NECT and AZU) than the offspring of predominately selfing plants. Such relations between a plant's outcrossing rate and the mean floral characters of its offspring could arise for two reasons. Offspring traits could vary directly with maternal outcrossing if the progeny of outcrossed mothers experience more heterosis than those of predominately selfed mothers. This direct effect seems not to be responsible for our results, as aspects of mean sibling flower size varied negatively, rather than positively, with maternal outcrossing rate. Alternatively, mean sibling floral traits could represent the heritable component of features of the maternal phenotype that affected the incidence of outcrossing by the maternal plants. Given our results, this latter explanation seems most likely, especially as the association of floral traits and outcrossing rates has a logical, functional interpretation. In particular, bees require longer to visit deep flowers and flowers with abundant and/or concentrated nectar (Harder, 1986), which, in turn, could increase the incidence of facilitated, intrafloral self-pollination.

Whether the relations between floral design and outcrossing apply generally for faba beans is uncertain.

Unlike our results, Pierre et al. (1996) found no association between self-fertility and nectar production in this species. Therefore, the effects of floral design on outcrossing may differ among populations, so that floral traits may not be the best targets for selection in breeding programs designed to enhance outcrossing.

Although aspects of floral design influenced outcrossing by faba bean plants, most of the variation in outcrossing, especially by nectar-producing plants, was associated with variation in floral display size (Table 2). Of particular importance, based on the standardized regression coefficients, were aspects of floral display during the first week of flowering. Specifically, maternal plants whose nectar-producing offspring displayed many open inflorescences (NNFA1), but few open flowers per inflorescence (NFA1) during the first week of flowering also had high outcrossing rates. Many studies have reported that bees visit plants with large floral displays more frequently and visit more flowers per inflorescence on large displays (reviewed by Ohashi & Yahara, 2001), which increases the incidence of self-pollination and reduces the pollen available for outcrossing (reviewed by Harder et al., 2004). By producing many inflorescences, each with relatively few flowers, plants in outcrossed families may attract more pollinators, while incurring less self-pollination than those in selfed families, especially if bees visit only a fraction of the available inflorescences.

The preceding interpretation initially seems incorrect for the results for all plants in the syn-4 population, as outcrossed families produced fewer inflorescence and more flowers per inflorescences than selfed families during the second week of flowering (Table 2). However, these results must be interpreted in the context of the additional significant display traits, because a partial regression coefficient identifies an independent variable's effect given the effects of other terms in the regression model. In particular, outcrossed families also produced more inflorescences, but fewer flowers during the entire flowering period than selfed families. Therefore, the significant display traits taken together suggest that outcrossed families produced a smaller proportion of their inflorescences and a larger proportion of their flowers during week two than did selfed families. This interpretation points to a specific, but unknown, feature about the mating environment during week two for the syn-4 population, which need not be inconsistent with the effects of display size on pollinator behaviour.

Our results provide little evidence that flowering phenology is an important determinant of outcrossing (Table 2). Maternal outcrossing did not vary significantly with either the mean first or last flowering date by offspring. Only the duration of the flowering period affected outcrossing significantly and only in the syn-4 population. That this relation was positive is consistent with Diggle's (1992) suggestion that increased autogamy may be a correlated response to selection for more rapid development.

Our demonstration that floral and inflorescence traits affect outcrossing by faba beans implies that outcrossing in this crop plant could be enhanced by artificial selection for appropriate aspects of floral design and display, without the use of male sterility. Traits deserving particular attention in such a selection program include enhanced inflorescence production during the beginning of flowering, but with few flowers open simultaneously per inflorescence. In addition, nectar production and flower depth warrant attention. Nectar-producing plants with low reward and short floral tubes *within the range that we examined* should promote outcrossing. Selection for decreased investment in nectar production may also allow plants to reallocate resources to ovules, providing an additional benefit to the plant. However, these recommendations are preliminary, because outcrossing by faba beans cultivars varies geographically (Suso & Moreno, 1999), so that the most suitable floral and inflorescence traits for promoting outcrossing may depend on local environmental conditions (Vogler et al., 1999), particularly the composition of the pollinator fauna. Further studies are needed to determine whether our results apply generally to other environments.

Effects of floral and inflorescence traits on yield and yield components

Like outcrossing, yield and its components varied significantly with aspects of floral design and display, but they were not affected strongly by flowering phenology (Tables 3 and 4). Almost all of the detected effects of reproductive traits on variation in yield among families involved the number of inflorescences open simultaneously, during either the fourth (final) week of flowering (syn-4 population), or the entire flowering period (syn-5 population: Table 3). Therefore, yield could be enhanced by selecting for plants with many open inflorescences. This suggests that floral traits promoting outcrossing may increase yield.

The two major components of yield, seed weight and seeds per plant, were influenced quite differently by floral and inflorescence traits (Table 4). Unlike seed

weight, which varied independently of design and display traits, seeds per plant depended on many of the same traits that affected overall yield (Table 4). This contrast indicates that selection on floral and inflorescence characters to increase yield should specifically focus on their effects on seed number. For nectar-producing plants, we also detected floral influences on seed production that were not evident in the analysis of overall yield. The signs and magnitudes of the partial regression coefficients for these effects indicate complex influences of floral traits, which were not consistent for the two synthetic populations. Interestingly, seed production varied negatively with nectar characteristics in the syn-4 population, as did the outcrossing rate. This negative relation is opposite to that reported by Teuber et al. (1990) for alfalfa. Nevertheless, the inconsistent effects of floral design on seed production among the two populations suggest that artificial selection would be better directed towards aspects of flower production and display.

The two main components of seeds per plant, pods per plant and seeds per pod were both influenced significantly by aspects of floral display and design (Table 4), although the specific influences differed between these components. Not surprisingly, pod production per plant varied positively with inflorescence production during the middle and latter half of the flowering season (NNFA2, NNFA3 and/or NNFA4) for both synthetic populations. After this effect of inflorescence production had been accounted for, total flower production (NTFA) affected pod production negatively for nectar-producing plants in the syn-4 population and for all plants in the syn-5 population. This result may indicate the effects of competition among developing fruits within infructescences. Pod production was also affected by a few aspects of floral design, but these effects differed among populations. This inconsistency in the effects of floral traits was also evident for average seed production per pod (Table 4); however, in this case the largely positive effects indicate, not surprisingly, that plants with larger flowers produce more seeds.

As with our analysis of the effects on outcrossing rate, we found that yield in these synthetic faba beans populations could be improved by increasing a plant's production of inflorescences, each with fewer flowers. This similarity could exist for two, non-exclusive reasons. First, the similar influences on outcrossing and yield could be a direct consequence of heterosis experienced by outcrossed families, but not by selfed families. Second, this similarity could exist because flower production and display govern outcrossing and yield via separate mechanisms (geitonogamy versus resource competition among fruit), which result in parallel responses. The extent to which outcrossing and yield are linked directly or exhibit correlated patterns of variation with floral display awaits further study.

General implications

Our study illustrates that floral design and display affect both outcrossing and yield and its components in faba bean, suggesting that yield potential, stability and outcrossing could be improved by selection on pre-mating reproductive characters. Accordingly, for faba bean breeding programs, the most effective approach to developing varieties that optimise yield in an environmentally sustainable manner would incorporate pre-mating reproductive traits, in addition to the customarily employed agronomic characters. Implementation of this approach requires more detailed information concerning additive genetic variation for floral design and display and the role of these traits in determining the mating system in different environments. Furthermore, the genetic association of these traits with each other and with other agronomic characters needs to be measured to determine whether correlated responses to selection would facilitate or inhibit the development of superior synthetic varieties. Nevertheless, it seems likely that a breeding program that incorporates an understanding of the function of floral design and display is more likely to produce environmentally sustainable varieties than one that does not.

Acknowledgments

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