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Estimation of Outcrossing Rate of Faba Bean under Natural Moroccan Conditions

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ABSTRACT

Background: Faba bean is a crop of agro-ecological and socio-economic importance in Morocco. Thus, in a changing climatic context, it is important to develop varieties that are resilient to several abiotic constraints, such as synthetics. However, the reproductive system must be understood.

Methods: We aimed to estimate faba beans outcrossing rate under natural Moroccan conditions, using two varieties, 'Reina Mora' and 'Zina' and two phenotypic markers, seed coat color and its size and shape, in two contrasted locations, Saïs and Gharb. Varieties were sown in six alternate rows with six seeds per row and three replicates per location. The experiment consisted in counting the number of purple seeds within 'Zina's beige offspring. Besides, seeds collected from 'Reina Mora' were counted and processed for differences in size and shape. In total, 859 seeds were measured, including their length, width, thickness, geometric diameter, area and sphericity, enabling to distinguish hybrids from parental seeds and self-seeds.

Result: Results showed an average inter-crossing of 3.7% in Saïs and 7.22% in Gharb. A more reliable open-pollination could be achieved by introducing bees and bumblebees colonies as well as using varieties from same geographic gene pools.

Key words: Allogamy, Autofertility, Broad bean, Cross-pollination, Synthetics.

INTRODUCTION

Vica faba L. is a globally important grain legume due to its ability to improve soil fertility, through symbiosis with *Rhizobium leguminosarum* in its root nodules and to its prominent role as a staple protein source in North African and Middle Eastern diets. However, faba bean acreage has fallen considerably over the past five decades in Morocco; it has decreased by more than half, from 278,400 ha to 125,860 ha between 1973 and 2019 (FAOSTAT, 2021). This gradual phasing-out is due to several climatic and technical constraints, but mainly due to the limited number of improved varieties. Thus, the few available varieties cannot adequately meet the demand of the different agro-ecological regions.

Faba bean Moroccan breeding program has been going on for more than 37 years and had a significant role in the improvement of seed yield and quality. To date, 25 varieties of faba bean major and 10 varieties of faba bean minor have been registered in the Official Moroccan Catalogue and are exclusively inbred lines. In order to create resilient varieties that are adapted to climate change, it would be interesting to exploit the hybrid vigor through the allogamous pathway of *Vicia faba* L. Unfortunately, breeding hybrid varieties is not possible because cytoplasmic male sterility (CMS) proved to be too unstable (Bond, 1987; Gasim and Link, 2007). An alternative strategy to exploit some of the heterosis present in faba beans could therefore be the development of synthetic varieties (Ebmeyer, 1988). Due to their genetic heterogeneity, synthetics serve to some extent as a buffer against unfavorable environmental conditions (Becker and Leon, 1988; Ebmeyer, 1988). Moreover, faba bean synthetic varieties were proven to be more productive, in terms of grain and biological yield, than both pure and bulk lines (Maalouf *et al*., 2018). Therefore, the crop reproductive system must be fully understood, because high cross-pollination levels lead to high heterozygosity and thereby to a high share of heterosis, resulting in high yield (Brünjes and Link, 2021).

The level of allogamy in *Vicia faba* L. varies considerably, ranging from 4 to 84% with an average of around 30-40% (Link *et al*., 1994; Suso *et al*., 2001). This variability depends on both genetic and environmental factors as well as the methods used for estimation (Link *et al*., 1994). Numerous studies have been conducted on the outcrossing rate in faba bean in France, Spain, Germany, Sudan, England and the United States of America (Picard, 1963; Suso *et al*., 2001; Gottschalk, 1960; Kambal, 1969; Rowlands, 1960; Hu *et al*., 2011, respectively). However, no assessment has taken place in Morocco. Thus, the present work aimed to estimate the outcrossing rate in *Vicia faba* L. under natural Moroccan conditions in the actual context of climate change. Two local

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varieties of faba bean major and faba bean minor were used under two contrasted environments.

The results provide a preliminary basis for ulterior open pollination breeding programs but also for climate change related studies, particularly the evolution of pollinator abundance in these two aforementioned regions.

MATERIALS AND METHODS

Plant material

Two varieties were chosen for this study; 'Reina Mora' (RM) and 'Zina' (Fig 1), based on their distinctive seed-related characteristics, namely testa color, shape and size of the seeds. 'Zina' is a Moroccan variety, developed by the National Institute of Agricultural Research (INRA) whereas 'Reina Mora' is Spanish developed by Semillas Fitó. Both varieties are registered in the Official Catalogue of Morocco. Reina Mora (major) seeds are purple, long, wide and almost flattened. In contrast, Zina (minor) seeds are beige, small and almost spherical. Seed coat color has been reported to be a morphological trait with simple and Mendelian inheritance (Ricciardi *et al*., 1985 and Mesquida *et al*., 1990). It is considered to be the most stable trait and least influenced by the environment (Picard, 1963). Prior to this study, no information on the level of cross-pollination has been reported on these varieties.

Experimental design

The experiment was conducted under open field conditions in the National Institute of Agricultural Research (INRA)'s experimental farms at Douyet (Saïs region) and Sidi Allal Tazi (Gharb region), during 2020-2021. These two environments are different in terms of pedoclimatic conditions. Each plot is installed on six rows at a rate of six seeds / row with an intra and inter-row spacing of 0.6 m. The elementary plot covers an area of 12.96 m^2 and is repeated three times per locality with a separation of 3 m. In order to prevent exogenous pollination from other faba bean plants, the trials were carried out in an isolated area far from any possible pollen source.

Parameters measured

Two phenotypic markers were used to identify the occurrence of cross-pollination between the4 two varieties, namely the color of the seed coat and the seeds' size and shape. Estimates were based upon the proportion of purple-seeded (dominant) progeny from beige-seeded parent 'Zina' (Ricciardi *et al*., 1985).

Regarding the quantitative marker 'size and shape', seed length, width and thickness were measured using a caliper. Measurements were taken on random samples, made up of 100 self-pollinated 'Reina Mora' offspring seeds from each replicate in addition to 100 seeds from each original parental variety, 'Reina Mora' and 'Zina'. These measurements provide information on seed sphericity, an important morphological trait that highly differentiates *V. faba* minor (high sphericity) from *V. faba* major (flattened seeds).

Seeds geometric mean diameter Dg and sphericity Φ were calculated using the following equations (Mohsenin 1970):

$$\Phi\ (\%) = \left(\frac{\sqrt[3]{LWT}}{L}\right)$$

$$Dg\ (cm) = \sqrt[3]{LWT}$$

The surface area of faba bean seeds was calculated using the following expression (Sacilik *et al*. 2003):

$$S\ (cm^2) = \pi Dg^2$$

Where,
L = Length (cm).
W = Width (cm).
T = Thickness (cm).

Estimation of outcrossing rate

Pigmentation polymorphism provides a convenient way to measure the outcrossing rate. In addition, this study is conducted in such a way that the two varieties are exploited. We tracked an additional marker to identify crosses within the so-called 'male' variety (dominant genotype), which is 'Reina Mora'. Erith (1930) indicates that the seeds of F1 progeny have an intermediate size between the two crossed parents with a shift to the small seeded female parent. Estimates of cross-pollination, commonly referred to by 't', were calculated, using the following expressions, at the level of the plots and the individual plants, which provides a better understanding of pollinators' behavior regarding each visited individual plant.

$$t\ \% = \frac{\text{Number of plants with hybrid seeds}}{\text{Total number of plants}} \times 100$$

$$t\ \% = \frac{\text{Number of pods with hybrid seeds per plant}}{\text{Total number of pods per plant}} \times 100$$

Statistical analysis

Parental genetic divergence

The genetic distance between parental lines is useful when using a quantitative genetic marker for natural outcrossing rate estimation. In order to ensure that the marker 'size and shape' is highly polymorphic and discriminates between the two varieties chosen, a mean comparison analysis, using Student test (α=0.05%), was carried out on a random sample

Fig 1: Reina Mora seeds (A) vs. Zina seeds (B).

of 100 seeds of each variety. The statistics software used is SPSS statistics 26.

Progeny analysis

In order to verify whether the pods which gave seeds of a distinct phenotype, in terms of size and shape, are effectively the results of crosses between *V. faba* major 'Reina Mora' and *V. faba* minor 'Zina', mean comparison analysis, using Student test was performed, using SPSS statistics software. Comparison was made between seeds resulting from the likely natural hybridization and the original parental seeds and between these same hybrids and the rest of the 'Reina Mora' offspring seeds.

RESULTS AND DISCUSSION

Parental genetic divergence

The comparison between the two parental varieties shows that the difference in terms of length, width and thickness is highly significant ($p<0.05$). Likewise, the t-test confirms that the sphericity of 'Zina' seeds is significantly higher than that of 'Reina Mora' (Table 1). Thus, the quantitative marker adopted is polymorphic and makes it possible to discriminate between the two varieties chosen.

Outcrossing rate estimation

Of the 108 plants sown in Sidi Allal Tazi (36 plants/replicate), eight plants of 'Reina Mora' (Plant 18 of the first replicate, plants 7, 8, 9 and 10 of the second replicate and plants 4, 12 and 14 of the third replicate) as well as three plants of 'Zina' (Plant 8 of the first replicate and plants 1 and 15 of the second replicate) did not survive, during pod formation period. In fact, 'Reina Mora' is more adapted to irrigated areas, which must have constrained its development, especially after the occurrence of several heat waves during 2021 growing season. Thus, the first replicate consists of 34 plants of both varieties, instead of 36, while the second and third plots are composed of 30 and 33 plants, respectively.

At the level of replicates, outcrossing rate ranged from 0 to 8.82% over the two locations. The highest average rate was observed in Sidi Allal Tazi (7.22%). In this environment and based on the 'seed coat color' marker, two plants of 'Zina' have been pollinated by 'Reina Mora', namely plant 9 (one flower) and 13 (4 flowers), spotted at the first and the second replicates respectively. The average rate of allogamy is then estimated at 2.06% over all the replicates (R1: 2.94%, R2: 3.33% and R3: 0%). The marker 'seed size and shape' clearly differentiates five plants of 'Reina Mora' collected in Sidi Allal Tazi. Plants 2 and 4 of the first plot, plant 1 of the second plot and plants 1 and 5 of the third plot (one flower each) showed a spherical shape that is very similar to that of faba bean minor 'Zina'. On the basis of the combined markers, seeds color, size and shape, the overall allogamy rate in Sidi Allal Tazi amounts to 7.22% (R1: 8.82%, R2: 6.66% and R3: 6.06%). In terms of individual plants, outcrossing rate in Sidi Allal Tazi varied from 1.66% to 25%. This rate is closely linked to the yield, mainly the total number of pods produced per plant. Overall, 'Zina' expresses a much higher pod yield than 'Reina Mora', which creates a significant gap in terms of cross-pollination rate in individual plants. With the exception of plant 13 of 'Zina', from the second replicate, which produced four hybrid pods, all of the plants ('Zina' and 'Reina Mora') had only one cross-pollinated flower by plant. The lowest outcrossing rate (1.66%) was observed on plant 9 of the first replicate recording a high yield of around 60 pods. On the other hand, the highest rate of allogamy (25%) was observed on plant 1 of 'Reina Mora' of the third replicate, due to the very low number of pods produced (four).

In Douyet, no purple coloration was observed in the seeds collected from 'Zina' plants. Therefore, the outcrossing rate based on the marker 'seed coat color' was zero.

Table 1: Comparison of the average seed size and shape between parental seeds of 'Reina Mora' and 'Zina'.

		Levene's test for equality of variances		t-test for equality of means				
		$F_{(1)}$	Sig. $_{(2)}$	$t_{(3)}$	$df_{(4)}$	Sig. (2-tailed)$_{(5)}$	Mean difference	Std. error of difference
Length	Equal variance assumed	0.504	0.479	17.938	198	0.000	2.722	0.152
	Equal variance not assumed			17.938	197.052	0.000	2.722	0.152
Width	Equal variance assumed			15.386	198	0.000	1.526	0.099
	Equal variance not assumed	26.149	0.000	15.386	167.348	0.000	1.526	0.099
Thickness	Equal variance assumed			-14.765	198	0.000	-1.734	0.117
	Equal variance not assumed	60.456	0.000	-14.765	146.878	0.000	-1.734	0.117
Dg	Equal variance assumed			-0.394	198	0.6940	-0.003	0.009
	Equal variance not assumed	27.076	0.000	-0.394	167.684	0.6940	-0.003	0.009
Φ %	Equal variance assumed			-16.624	198	0.0000	-10.202	0.614
	Equal variance not assumed	21.786	0.000	-16.624	153.008	0.0000	-10.202	0.614
S	Equal variance assumed			-0.561	198	0.5750	-0.035	0.062
	Equal variance not assumed	27.532	0.000	-0.561	166.754	0.5760	-0.035	0.062

(1); Levene's F Test for Equality of Variances, (2); Significance level, (3); Standard t-test, (4); Degrees of freedom, (5); p-value for two-tailed tests.

However, four plants of 'Reina Mora', 5 and 12 from the first replicate and 1 and 18 from the second one produced one pod each, containing similar seeds to faba bean minor 'Zina' in terms of size and shape (Fig 2). The natural hybridization rate in Douyet is therefore equivalent to 3.70% (R1: 5.55%, R2: 5.55% and R3: 0%), which is half the rate recorded in Sidi Allal Tazi. The outcrossing rate of individual plants in Douyet varied from 6.25% to 10%. This rate is relatively high due to the low number of pods (10 to 16) produced by 'Reina Mora'.

The overall outcrossing frequency observed in this experiment is low, compared to the average rate 40-50% (Bond and Poulsen, 1983). Early studies reported quite different results. For instance, Drayner (1959), Rowlands (1960), Fyfe and Bailey (1951) in England, Picard (1963) in France, Gottschalk (1960) in Germany and Kambal (1969) in Sudan reported high outcrossing rates, largely exceeding 30% and, therefore, granted *Vicia faba* L. an intermediate position between allogamy and autogamy, whereas Muratova (1931) admitted that intercrossing could be significantly curtailed by a simple isolation. Likewise, Lechner (1962) reports low outcrossing rates, 8-10% in Germany and considers that isolation is sufficient for line fixation and varietal purity maintenance.

Numerous factors could limit outcrossing rates. In fact, synchrony in flowering periods was taken into account when choosing the plant material. However, the highly selfing nature of the two lines 'Zina' and 'Reina Mora', could tend to induce a gametic selection for autopollen over allopollen (Mesquida *et al*. 1990). It is known that in preferential selfing species, for which inbreeding depression is weak, low levels of outcrossing are often found (David *et al*., 1993). Thus, the use of self-fertile material should be preferably avoided in breeding synthetics varieties, as recommended by Becker (1988). Another possible explanation has been put forward by Ibarra-Perez *et al*. (1997) who reported that the use of parental germplasm from different geographic gene pools may give lower estimates of outcrossing rate than use of germplasm from the same gene pool. As a matter of fact, the variety 'Zina' is Moroccan whereas 'Reina Mora' is Spanish.

Weather conditions might also affect natural hybridization. It is important to note that the 2020-2021 growing season was a singular year, thanks to the heavy rainfall that spread over a long period (from October to May). The peak of precipitation, *i.e.* 113 mm was recorded in April at the experimental farm of Douyet which coincides with faba bean flowering season, suggesting that continuous rainfall sequences might have limited bees activity during this critical period. In addition, the variation in the abundance of flower-visiting species, either spatially or seasonally, has a major influence on cross-pollination within and among crop fields, hence the difference spotted between the two tested regions, Sidi Allal Tazi (Gharb, 7.22%) and Douyet (Saïs, 3.70%). These findings were in agreement with our primary assumption in the field, owing to the fact that the experimental farm of Douyet was strangely and unusually devoid of bees and bumblebees.

The decline in bees during the bloom period aroused our curiosity. Therefore, we launched an investigation with the Moroccan Interprofessional Federation of Beekeeping (FIMAP) to understand and supplement our results. No cases of mass death have been reported in the Gharb region apiaries, including Sidi Allal Tazi. However, the decline of honeybee populations is much more pronounced in Saïs region where massive colony losses occurred subsequent to an invasion of the varroa mite parasite, in conjunction with lax control. Fes-Meknes region beekeepers do not treat or little with varroacides or alcohol washes. Instead, they use plants consumed in a smoker, the effectiveness of which is not known (Mohssine *et al*., 2020). It is also important to not overlook the repercussions of climate change that modifies plants spatial distribution and their biological cycles (with a possible desynchronization of the plant biology and the bees foraging activity) and therefore creates less favorable survival conditions for pollinators on some territories (Soroye *et al*., 2020).

Our results were in agreement with those of Mesquida *et al*. (1990) where outcrossing rate under natural conditions, at INRA of Rheu in France was 5.1%, using the same marker, seed color. They reported that cross-pollination, after bees introduction, barely reached 9.4%. Cages with bumblebees recorded the highest rate, 11.6%, which is still much lower than average 40-50% (Bond and Poulsen, 1983). Contrasting results were found by Suso

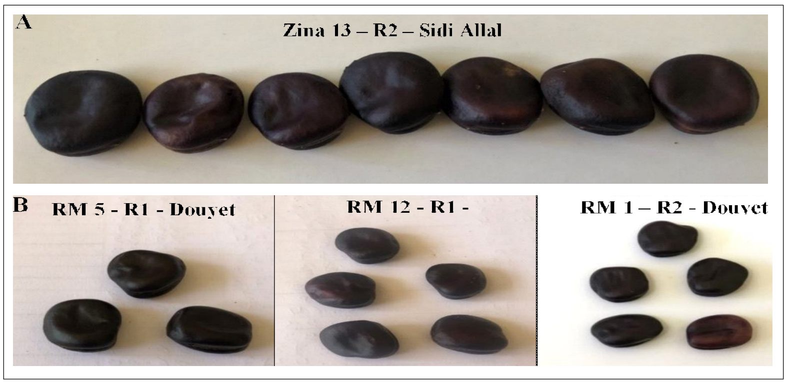

Fig 2: A; 'Zina' hybrid seeds profile, B; 'Reina Mora' hybrid seeds profile.

et al., (2001) in Rennes, France and in Cordoba, Spain, where allogamy rates reached 33% and 65%, respectively. Similarly, higher rates (30.8%) have been published by Hu *et al*. (2011) using flower color as a genetic marker, in Pullman, United States.

Comparison between the shape and size of 'Reina Mora' F1 hybrid seeds and the parent

The t-test results confirmed that seeds size and shape differed significantly between the parent and the F1 progeny resulting from natural hybridization ($p < 0.05$), except for the width (Table 2). Given the relevance of the character 'sphericity' in the differentiation between faba bean major and minor, it is important to highlight the difference observed between 'Reina Mora' parental seeds and the F1 hybrid seeds resulting from the natural crossing with 'Zina'. Indeed, the hybrid seeds are 70.8% spherical while the parental seeds exhibit a more flattened appearance with an average sphericity of 59%. This difference is illustrated in a box diagram (Fig 3).

Comparison between the shape and size of 'Reina Mora' F1 hybrid seeds and the self-pollinated seeds

In the same perspective, the t-Student test was carried out to check whether there are significant differences between the F1 seeds obtained from the likely natural hybridization and the seeds produced by selfing. As expected, the two types of seeds are significantly different in terms of length, width, thickness, geometric diameter, sphericity and surface area (Table 3). Seeds sphericity represents a differentiating trait. Thus, it is important to note that the sphericity of F1 hybrid seeds is around 70.8% while those derived from self-

Table 2: Comparison of the average seed size and shape of 'Reina Mora' between parent and F_1 hybrids.

		Levene's test for equality of variances		t-test for equality of means				
		$F_{(1)}$	Sig. $_{(2)}$	$t_{(3)}$	$df_{(4)}$	Sig. (2-tailed)$_{(5)}$	Mean difference	Std. error of difference
Length	Equal variance assumed			9.835	135	0.000	2.4224	0.2463
	Equal variance not assumed	8.931	0.003	7.773	45.231	0.000	2.4224	0.3116
Width	Equal variance assumed			-2.638	135	0.009	-0.5545	0.2102
	Equal variance not assumed	42.661	0.000	-1.729	38.043	0.092	-0.5545	0.3207
Thickness	Equal variance assumed			-7.614	135	0.000	-1.4530	0.1908
	Equal variance not assumed	8.579	0.004	-5.089	38.615	0.000	-1.4530	0.2855
Dg	Equal variance assumed			-3.414	135	0.001	-0.0515	0.0151
	Equal variance not assumed	22.886	0.000	-2.356	39.624	0.023	-0.0515	0.0219
Φ %	Equal variance assumed			-15.142	135	0.000	-11.8182	0.7805
	Equal variance not assumed	12.144	0.001	-11.202	42.189	0.000	-11.8182	1.0550
S	Equal variance assumed			-3.737	135	0.000	-0.4074	0.1090
	Equal variance not assumed	26.661	0.000	-2.552	39.276	0.015	-0.4074	0.1596

(1); Levene's F Test for Equality of Variances, (2); Significance level, (3); Standard t-test, (4); Degrees of freedom, (5); p-value for two-tailed tests.

Table 3: Comparison between the shape and size of 'Reina Mora' F1 hybrid seeds and the self-pollinated seeds.

		Levene's test for equality of variances		t-test for equality of means				
		$F_{(1)}$	Sig. $_{(2)}$	$t_{(3)}$	$df_{(4)}$	Sig. (2-tailed)$_{(5)}$	Mean difference	Std. error of difference
Length	Equal variance assumed			19.916	635	0.000	4.345	0.218
	Equal variance not assumed	5.143	0.024	14.565	38.207	0.000	4.345	0.298
Width	Equal variance assumed			4.746	635	0.000	0.925	0.195
	Equal variance not assumed	17.792	0.000	2.897	37.430	0.006	0.925	0.319
Thickness	Equal variance assumed			-9.889	635	0.000	-1.070	0.108
	Equal variance not assumed	35.144	0.000	-3.805	36.394	0.001	-1.070	0.281
Dg	Equal variance assumed			4.607	635	0.000	0.0528	0.011
	Equal variance not assumed	27.281	0.000	2.456	37.017	0.019	0.0528	0.021
Ö %	Equal variance assumed			-24.738	635	0.000	-12.283	0.496
	Equal variance not assumed	45.471	0.000	-12.055	36.802	0.000	-12.283	1.019
S	Equal variance assumed			4.171	635	0.000	0.3599	0.086
	Equal variance not assumed	25.117	0.000	2.287	37.094	0.0280	0.360	0.157

(1); Levene's F Test for Equality of Variances, (2); Significance level, (3); Standard t-test, (4); Degrees of freedom, (5); p-value for two-tailed tests.

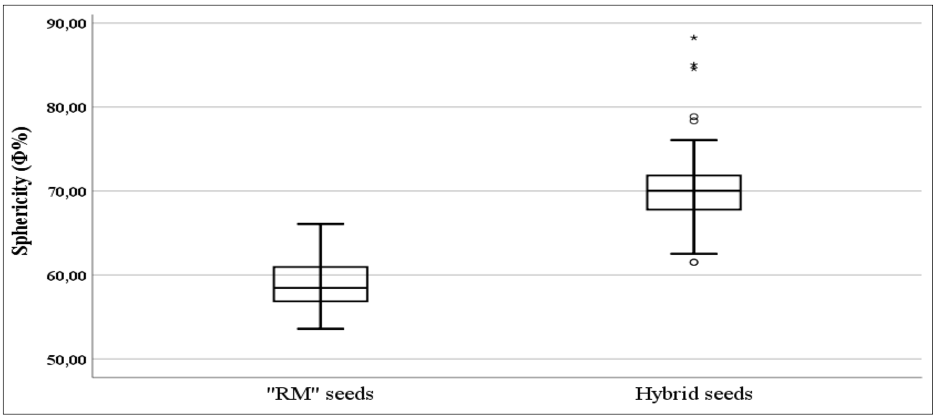

Fig 3: Difference in terms of sphericity between two samples of unequal sizes: Parental seeds of 'Reina Mora' (RM) and F_1 seeds from natural hybridization with 'Zina'.

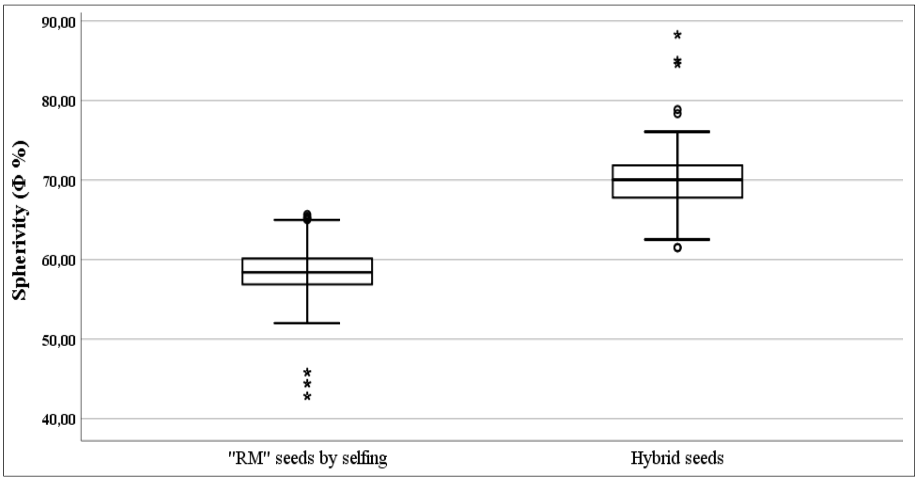

Fig 4: Difference in terms of sphericity between two samples of unequal sizes: Seeds of 'RM' from self-pollination and F1 seeds from natural hybridization.

pollination show an average sphericity of 58.5%; this difference is illustrated in Fig 4.

CONCLUSION

As a partially allogamous crop, faba bean has a wide range of outcrossing rate, because of various factors, such as pollinators, environment, geographical factors, population density, genotype, size of inflorescence and flower structure. Variation in outcrossing is worthy of regional studies as it is important to define breeding strategies. In this case, inter-crossing rate varied across locations. It reached 7.22% in Sidi Allal Tazi (Gharb) and stood at 3.70% in Douyet (Saïs).

Given Morocco's tendency towards inbred lines development, these findings are less worrying and allow a convenient and more reassuring conduct of classic breeding programs aimed at self-pollinated lines development. Simple spatial isolation may spare efforts and time. However, before considering synthetic varieties, these estimates are in fact very informative. The use of self-fertile material should be preferably avoided as well as varieties from different geographic gene pools. Moreover, pollinator decline is a global reality today. When breeding synthetics, better and more reliable pollination could be achieved by introducing managed colonies of bees and bumblebees.

Conflict of interest: None.

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