

# Efficiency of hand pollination in different pea (*Pisum*) species and subspecies

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(Received: May 2013; Revised: September 2013; Accepted: November 2013)

## Abstract

With a view to optimise the hand pollination procedures for different pea species and subspecies, eight accessions belonging to *Pisum fulvum*, *Pisum sativum* ssp. *elatius* (wild), *P. abyssinicum* and *P. sativum* ssp. *sativum* (cultivated) were used to compare the mean number of seeds per crossed flower (crossing efficiency) using three methods of pollination: 1) immediately after emasculation of a flower bud before the anthers burst; 2) after opening of the flowers emasculated as above (delayed pollination) and 3) double pollination combining the first two methods. In the cultivated species, the seed setting did not differ much between the methods. In wild peas, single pollination after flower opening gave similar or better results than single pollination immediately after emasculation. Double pollination in most cases gave results comparable to single pollination after flower opening. In accession J13262 of *Pisum sativum* ssp. *elatius* with exceptionally large flowers, single pollination after flower opening appeared twelve times as efficient as pollination after emasculation. Delayed pollination may be recommended for crossing wild peas.

**Key words:** Wild pea, hand crossing method, selfing, crossing-efficiency

## Introduction

Hand pollination was one of the first experimental techniques in genetics. Mendel [1] spent years in methodical preparation of his experiments including choice of peas as experimental plants and testing the crossing procedure, which did not change since Mendel's times. The keel is cut or removed before the corolla gets pigmentation and opens, the anthers are removed by cutting stamen threads with pincers, and pollen is put onto the stigma; no protection from further

pollination. This implies two assumptions: (i) the pistil becomes competent to pollination before the corolla is open, and (ii) after the corolla gets open, further pollination is improbable. These assumptions imply pea to be a strict self-pollinator. Indeed, in pea, the anthers usually burst out at the stage when a flower bud gets anthocyanin coloration but yet did not open. However, pea flowers attract Apidae insects so that occasional cross-pollination does occur, up to 30% of progeny in some pea forms of southern origin [2]. Administration of alien pollen onto stigmata of open pea flowers showed that the pistil retains competence to pollination at this stage, with some ovules not yet pollinated by own pollen [3].

When we crossed diverse peas to test their compatibility (under preparation) the results hinted that the standard crossing procedure may not fit best to some wild forms. To exclude effects of cross incompatibility we compared hand pollination of eight divergent pea forms by their own pollen with three different methods.

## Material and methods

Eight homogenous accessions were used representing divergent pea genotypes (Table 1).

The plants were grown in greenhouse (in claydite/vermiculite hydroponic beds fed twice a day by Knop nutrient solution; under 8 h day light/16 h incandescent light of 10,000-12,000 lux intensity) during January-April 2010, October-December 2010, and March-May

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**Table 1.** Pea accessions, their flower characteristics, the mean and maximum number of seed in pods formed on selfing

Accession	Origin	Flower features	Mean seed no./ pod formed on selfing	Max. seed no. formed on selfing
WL2140 <i>Pisum fulvum</i> Sibth et Smith, a wild species from Anterior Asia	Israel, valley of cross	1-2 per peduncle, small, fully open, standard short	2.85±0.06 n = 315	5
VIR2759, <i>Pisum abyssinicum</i> A. Br., cultivated in Ethiopia and Yemen	Ethiopia	1 per peduncle, small	4.08±0.17 n = 64	7
J13273 (=712 in [7]) <i>Pisum sativum</i> ssp. <i>elatius</i> (Bieb.) Schmahl. s. l., a wild subspecies from Mediterranean	Israel, 10 km S of Be'er Sheva	1, rarely 2 per peduncle; pedicle skewed at an angle to peduncle axis small, fully open, standard rather short	3.62±0.21 n = 37	6
VIR320, <i>P. sativum</i> spp. <i>elatius</i>	Palestine	1, rarely 2 per peduncle, shortening on higher nodes medium sized, fully open	4.87±0.11 n = 239	9
J13262 (= 721 in [7]), <i>P. sativum</i> spp. <i>elatius</i>	Israel, Mt. Carmel, 5 km NE of Zikhon Ya'akov	1, on very long, arch-like peduncle, large, fully open, standard unproportionally large, about 3.5 cm wide	4.06±0.36 n = 32	7
J11794, <i>P. sativum</i> spp. <i>elatius</i>	Golan Heights, Tel Abu Nida	1, on very short peduncle, small, wings narrow; standard not fully open, rolled so that the flower is widest at its base, pigmentation very weak	3.70±0.29 n = 36	6
J12629 (=CE1), <i>P. sativum</i> spp. <i>elatius</i>	Crimea, Simeiz	1, on short peduncle, small, looks narrow and pointed; wings narrow; standard not open and rolled, pigmentation very weak	2.75±0.14 n = 110	6
WL1238 <i>Pisum sativum</i> ssp. <i>sativum</i> L., cultivated worldwide	A tester line	2, rarely 1 on peduncle, very short, alae reduced ( <i>kk</i> ); anthers burst out very early; pigmentation nearly absent ( <i>kk bb</i> )	no statistical data	8

n = number of pods

2012, hereafter referred to as “spring 2010”, “autumn 2010” and “spring 2012”, respectively.

The flower buds were emasculated by cutting the keel along its length and removing the anthers with forceps several hours before appearance of anthocyanin pigmentation. Accessions J11794 and J12629 have mild pigmented flowers. Their flower buds were emasculated at the designated stage as judged from their shape and development. They were pollinated with the pollen taken from open flowers of the same accession by forceps using three methods:

Method 1: immediately after emasculatation (the standard method).

Method 2: when the corolla gets fully open and coloured (delayed pollination). This happens the next day after emasculatation in all but accession J13262. In this genotype with very large flowers, anthesis takes place a day later, so they were crossed the second day after emasculatation.

Method 3: twice, as in methods 1 and 2 combined (double pollination).

All three methods were applied simultaneously on the same plants taking different flower buds randomly. In spring 2010 and spring 2012, at least 30 crosses were made. Seeds were collected individually from each pod in spring 2010 and 2012, but the pods

were pooled for each method in each accession in autumn 2010.

For each accession and crossing method, the number of pods set and seeds developed as well as the mean number of pods formed per cross, number of seeds per pod and per cross were counted. The latter parameter reflects the crossing efficiency.

Values of the parameters were compared for the three crossing methods applied to the same accession in the same season. The mean number of pods per cross is an estimation of the probability of the pod formation after a cross. Significance of differences in this parameter was tested using the Welsh t-test [5] after application of Fischer's  $\epsilon$ -transformation [4]. The mean number of seeds per pod is the probability of fertilization multiplied by the number of ovules in a pod, which is a quantitative trait depending on continuous factors but rounded off to integer values. The mean number of seed per cross is a product of the two previous parameters and therefore is determined by superposition of random events of different nature. When pods were collected individually we estimated the variance for the number of seeds per pod and per cross and evaluated differences with Welch t-test ( $t_w$ ).

In spring 2013, the number of seeds obtained from naturally self-pollinated flowers in isolation (in a greenhouse free from pollinators) was counted in the pods individually, their mean number with the standard errors is given in Table 1.

## Results and discussion

The estimates of the parameters for each accession are presented in Table 2. For the crosses made in autumn 2010, we missed data on the seed number in individual pods. Note, however, in each accession except JI3262 and JI2629, the standard deviations of the number of seeds per cross varied quite moderately between crossing methods. So, for statistical comparisons involving the data of autumn 2010, we adopted the highest value of each variance (to avoid overestimation of differences) of each parameter obtained for the relevant accession in spring 2012. For instance, for for VIR2759 autumn 2010 we assume  $\sigma_e$  to be 1.76 for all three methods. For JI1794 we have no spring 2012 data and assume both  $\sigma_s$  and  $\sigma_e$  to be 2.00 (a bit higher than the values of  $\sigma_e$  observed in for VIR2759 showing very similar values of  $s$  and  $e$ ).

The mean number of pods per cross varied little for the pollination methods in most accessions. For VIR320, it was nearly twice as much for delayed pollination than for the two other methods, the differences being highly significant ( $t = 4.16$  and  $3.86$ , respectively;  $P < 0.001$ ). Accession JI3262 appeared exceptional showing one-fourth the value for immediate pollination (0.11), in which only four pods were formed in 38 crosses attempted, as compared to the one for delayed (0.40) and double (0.44) pollination, the differences being highly significant ( $t = 2.94$  and  $3.01$ , respectively,  $P < 0.01$ ). Two other cases of significant differences (at  $P < 0.01$ ) and moderate in magnitude (1.5 times) were observed in spring 2012 but not in the analogous experiments earlier, in autumn 2010: a higher value with immediate pollination than with double pollination in WL2140, and a higher value with delayed pollination than with the two other methods in VIR2759.

In most cases, the mean number of seeds per pod also varied to a less extent among the pollination methods. This means that (i) if a pod with seeds is formed, the number of seeds only slightly depends on the time of pollination, and (ii) the second pollination neither damages the pistil nor adds seeds. The strongest effects, at 1% significance level, approximately 3.5-fold and 4.5-fold increase in the mean number of seeds per pod with delayed and double pollination, respectively, as compared to immediate pollination, were observed in accession JI3262. Effects of the same magnitude but less significant (at 5% significance level in case of immediate versus double pollination) were observed in VIR320. The small effects at 5% significance level in WL2140 and JI3273 were not reproducible over seasons: in case of WL2140 slight superiority of delayed pollination over immediate pollination was not statistically significant in spring 2012, while in JI3273, immediate pollination was significantly inferior to double pollination in autumn 2010 and to delayed pollination in spring 2012.

It was interesting to compare the mean number of seeds in a pollinated pod (Table 2) with the mean number of seeds in the selfed pods (Table 1). The latter is much smaller than the maximum number of seeds that can be formed in a pod (Table 1). For most of the accessions, the mean number of seeds per pod after crosses at least for some pollination method(s) was comparable to the number of seeds set on natural selfing. For JI2140, VIR320, JI3273 (spring experiment), JI3262 some of the former values even

**Table 2.** Comparison of different crossing methods immediate pollination (method 1), delayed pollination (method 2) and double pollination (method 3) performed during 2010 and 2012

Accession	Method	No. of crosses	No. of pods formed	No. of seeds formed	Mean no. of pods/cross	Mean no. of seeds/pod	Crossing efficiency: mean no. of seeds/cross	Standard error of crossing efficiency
WL2140 Autumn 2010	1	42	32	81	0.76	2.53 <sup>*2</sup>	1.93 <sup>*2</sup>	-
	2	30	27	87	0.90	3.22	2.90	-
	3	39	32	91	0.82	2.84	2.33	-
WL2140 Spring 2012	1	38	33	90	0.87 <sup>**3</sup>	2.73±0.20	2.37±0.23	1.40
	2	36	28	86	0.78	3.07±0.22	2.39±0.28	1.66
	3	38	22	71	0.58	3.23±0.25	1.87±0.30	1.85
VIR2759 Autumn 2010	1	41	21	75	0.51	3.57	1.83	-
	2	35	16	43	0.46	2.69	1.23 <sup>*3</sup>	-
	3	27	19	57	0.70	3.00	2.11	-
VIR2759 Spring 2012	1	37	19	32	0.51	1.68±0.46	0.86±0.27	1.65
	2	36	29	58	0.81 <sup>**1,3</sup>	2.00±0.33	1.61±0.29	1.76
	3	30	17	19	0.56	1.12±0.36	0.63±0.23	1.25
JI3273 (712) Autumn 2010	1	54	33	79	0.61	2.39 <sup>*3</sup>	1.46 <sup>**3</sup>	-
	2	34	25	79	0.74	3.16	2.32	-
	3	48	40	141	0.83	3.52	2.94	-
JI3273 (712) Spring 2012	1	37	25	86	0.68	3.44±0.29 <sup>*2</sup>	2.32±0.33 <sup>**2</sup>	2.03
	2	30	28	122	0.93	4.36±0.34	4.07±0.37 <sup>**3</sup>	2.05
	3	38	27	103	0.71	3.81±0.26	2.71±0.34	2.09
JI1794 Autumn 2010	1	30	15	18	0.50	1.20	0.60	-
	2	33	23	25	0.70	2.24	0.76	-
	3	29	17	38	0.59	1.09	1.31	-
JI3262 (721) Spring 2010	1	38	4	4	0.11 <sup>***2,3</sup>	1.00±0.41 <sup>**2,3</sup>	0.11±0.06 <sup>**2,3</sup>	0.39
	2	34	13	45	0.40	3.46±0.70	1.32±0.39	2.29
	3	25	11	49	0.44	4.46±0.87	1.96±0.58	2.92
JI2629 (CE1) Spring 2010	1	34	25	31	0.74	1.24±0.41	0.91±0.31	1.82
	2	30	23	31	0.77	1.35±0.26	1.03±0.23	1.25
	3	26	20	22	0.77	1.10±0.26	0.85±0.22	1.12
VIR320 Spring 2010	1	34	16	41	0.47	2.56±0.44 <sup>*3</sup>	1.21±0.30 <sup>***3</sup>	1.75
	2	37	18	70	0.90 <sup>***1,3</sup>	3.89±0.62	1.89±0.44 <sup>**3</sup>	2.66
	3	30	27	113	0.49	4.19±0.40	3.77±0.43	2.34
WL1238 Spring 2010	1	26	18	48	0.69	2.67±0.29	1.85±0.32	1.62
	2	28	18	44	0.64	2.44±0.45	1.57±0.37	1.93
	3	31	25	68	0.81	2.72±0.30	2.19±0.31	1.72
WL1238 Spring 2012	1	36	23	59	0.64	2.57±0.24	1.64±0.26	1.55
	2	34	27	83	0.79	3.07±0.37	2.44±0.36	2.11
	3	28	20	57	0.74	2.85±0.35	2.04±0.35	1.86

After ±, standard error is given; \*, \*\* and \*\*\* stand for P<0.01, P<0.01 and P<0.001 for differences in crossing efficiency values between methods, lowercase figures indicate which method was compared (e. g. <sup>\*\*\*2,3</sup> at the value at method 1 refers to significance of its differences as compared to methods 2 and 3)

exceeded the latter ones. Only in JI1794 the greatest value of the mean number of seeds per pod after crossing for method 2, was only 60% of the mean number of the seeds set on selfing; and for JI2629 the greatest value of the former parameter for method 2 was 71% of the latter parameter. The mean numbers of seeds per pod after crossing and after natural selfing were not strictly comparable as they were recorded in different years, yet the data suggest that for each accession, at least one of the three crossing methods resulted in sufficient pollination.

The crossing efficiency, as measured by the mean number of seeds per cross, demonstrated significant differences between methods for five accessions (JI2140, VIR2759, JI3723, JI3262, VIR320) and none for accessions JI1794, JI2629 and WL1238. However, when the same accessions were used in crosses both in autumn 2010 as well as in spring 2012, the result were reproduced only for WL1238. In JI2140 and VIR2759, the statistically significant effects observed in autumn 2010, but were not observed in spring 2012. In JI3273, some differences were highly significant but not reproducible in both experiments: the crossing efficiency for immediate pollination was approximately twice as high as that for double pollination in autumn 2010 while in spring 2012 these methods showed similar values but that for delayed pollination was about 1.5 times higher. In VIR320, the crossing efficiency increased from immediate pollination to delayed pollination, and from delayed pollination to double pollination, the increase from immediate pollination to double pollination being almost three-fold, and the differences of the results with double pollination from those with the two other methods being highly significant.

The strongest effect was observed in accession JI3262, in which the crossing efficiency exhibited more than ten-fold increase with immediate pollination as compared to delayed pollination (from 0.11 to 1.32,  $t = 3.04$ ,  $P < 0.01$ ) and increased about 1.5 times more, although not significantly ( $t = 0.91$ ,  $P < 0.3$ ) with double pollination.

The mean number of pods per cross cannot be strictly interpreted. A pod may not be formed for two reasons: (i) if no ovule is fertilized, although small empty pods often appear (especially frequently in VIR2759, JI2629, and JI1794; counted as pods with 0 seeds), and (ii) if the pistil was mechanically damaged. However, the most injurious procedure was

emasculation and it was carried out identically in all methods. Pollination is a more gentle procedure but it differed between the methods. We may expect the damage during pollination to be greater with immediate or double pollination, when a more subtle young pistil is touched, than with delayed pollination. In VIR320 we face the situation when the mean number of pods per cross was much greater with delayed pollination than with the two other methods; smaller differences of the same kind were observed also in VIR2759 in spring 2012, but not so in other accessions. A four time smaller value of the mean number of pods per cross with immediate pollination as compared to delayed and double pollination in JI3262 cannot be interpreted in terms of mechanical damage and probably resulted from fertilization failure.

The mean number of seeds per pod, that is crossing efficiency, is the most important parameter. It is a product of the mean number of pods per cross and the mean number of seeds per pod, and could accumulate variation of both parameters. Our data suggest, however, that the significant differences in the crossing efficiency were concordant to those in the mean number of seeds per pod, often with a somewhat greater significance, but not to those in the mean number of pods per cross (WL2140 and VIR2759 in spring 2012 showed significant differences in the mean number of pods per cross but not in that of seeds per cross). This may be interpreted in the way that the crossing efficiency is limited mostly by pollination failure rather than the pistil damage.

As to crossing efficiency, we had the following observations:

- if it is greater with immediate and double pollination than with delayed pollination, the pistil competence is maximum in a still closed flower bud and a genotype is a self-pollinator;
- if it is equal with all the methods, ovules become receptive in a still closed flower bud and, if not fertilized, retain receptivity in an open flower; a genotype is a self-pollinator;
- if it increases from immediate to delayed pollination but not from delayed to double pollination, the time window of receptivity of each ovule is short but different ovules become receptive during a prolonged period of time from a closed to open flower and an accession combines selfing and outbreeding;

- if it is greater with delayed and double pollination than with immediate pollination, the pistil competence is maximum in an open flower and an accession is predominantly an outbreeder.

A meager reproducibility of the results for three (WL2140, VIR2759 and JI3273) of the four accessions for which repeated experiments were conducted suggests that crossing efficiency may depend on some factors which we still cannot account for. In the autumn greenhouse season the plants get less illumination due to the reduced sunshine at daytime, in the spring generation the temperatures may rise very high (perhaps the most important factor in our case). Because of that we shall pay attention only to strong effects observed in the crossing efficiency.

Our experiment involved only two cultivated but ..... *P. sativum* ssp. *sativum* cultivated worldwide (WL1238) and *P. abyssinicum* (VIR2759) endemic to Ethiopia and Yemen. In them, no difference was observed between the three methods, except for some drop in the crossing efficiency with delayed pollination in VIR2759 in the autumn 2010 experiment. This means that the two accessions are strict selfers, an expectable result of an unconscious selection under cultivation, with shortage of insect pollinators. Hence the standard crossing procedure fits the cultivated pea best.

Other six accessions represented wild peas. WL2140 belongs to a clear-cut species *Pisum fulvum*, other five are wild representatives of *P. sativum*, presently united into a loose subspecies *P. sativum* ssp. *elatius* in a broad sense. In all of them and in all cases, delayed pollination resulted in higher crossing efficiency than immediate pollination, although in JI2629 (a single trial in spring 2010), WL2140 in spring 2012 and JI3273 in autumn 2010, this effect was insignificant. Where significant, the effect means that the pistil retains competence for pollination after the flower is open, which implies a possibility of spontaneous cross-pollination which may be advantageous in natural conditions. This concerns accessions, JI3262, VIR320, and maybe JI2140 and JI3273, where the effect was milder and was not reproduced in one of the two experiments. In JI3262 and VIR320, the crossing efficiency increased from

immediate pollination through delayed pollination to double pollination, although not in all cases the differences were significant.

Accession JI3262 representing a tall climbing form [6] is exceptional in that its pistil is competent to pollination mostly after flower opening. This well correlates with exceptional flowers of this accession: very large with especially large standard, disposed on very long peduncles. No doubt this wild pea form is specially adapted to cross-pollination.

Although the study in wild peas showed somewhat different results, delayed pollination may be recommended for crossing any wild pea, as showing either the same or better results than the standard crossing procedure, immediate pollination. Double pollination, which is a combination of immediate and delayed pollination, is more laborious and appeared to be nearly as effective in most cases. Immediate pollination could be recommended for cultivated peas.

#### Acknowledgement

This work was supported by the project VI.53.1.3. "Genetic control of mechanisms of incompatibility between plant taxa and their adaptation to unfavourable environmental conditions".

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