Heritability and phenotypic variation of double seeds in almond (*Prunus dulcis*)

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Running title: **Heritability of double seeds in almond**

Abstract

In almond, the presence of double seeded nuts significantly reduces their commercial value. For this reason, in breeding programs of this species, seedlings producing double seeds are usually eliminated. In order to avoid this trait in the offspring and increase the efficiency of the breeding programs it is important to know its genetic control. In this work, heritability of double seeds was estimated by midparent-offspring regression (narrow sense, h^2) and by variance components analysis (broad sense, H^2) for three years, in a population with a combination of full-sib and half-sib offspring coming from eight crosses designed for this objective. Heritability was high by both methods every year, and the values calculated by variance components were higher than those estimated by regression. The average ratio h^2/H^2 0.69 implies some influence of a non-additive effect for this trait, such as dominance and environmental effect. In this case, the influence of the temperatures preceding blooming on the percentage of double seeds can be inferred from our results, with lower temperatures increasing the percentage of double seeds. In the crosses between parents with high percentage of double seeds a normal (Gaussian) phenotypic distribution was observed. For crosses between one parent without double seeds and parents with high-medium percentage of double seeds a right-skew distribution (L-shaped) was observed, and certain transgressive segregation was observed in the descendants. This transgressive segregation was confirmed when both parents presented null percentage of double seeds. The results confirmed the complex architecture of this trait transmitted quantitatively, where additive, non-additive and genotype-by-environment interaction effects play an important role.

Keywords: *Prunus dulcis*, almond breeding, double seeds, inheritance, quantitative trait.

Introduction

In most *Prunus* fruit tree species such as apricot, peach, cherry or plum, the edible part of the fruit is the mesocarp. Almond [*Prunus dulcis* (Miller) D.A. Webb] is an exception; the seed is the edible part of the fruit and the mesocarp is just utilized as livestock feed. Therefore, one of the main objectives for breeders in almond breeding programs is to obtain seeds of high quality that fit the different commercial requirements of the industry, which depend on destination (fresh, roast, fried, nougat, etc.), and consumers. In general, good shape, sweet and big single seeds are desired. Indeed, the presence of double seeds is undesirable for both industry (since double seeds are deformed they present difficulties for blanching and their commercial value is significantly lower), and consumers (because they are less attractive than single seeds). However, since double seeded nuts are heavier than single seeded ones from the same cultivar, the presence of a high number of double seeded nuts will increase the final production of a tree.

Understanding the advantages and disadvantages of a specific trait is an important task for almond breeders, who must design optimal breeding strategies to obtain the best response to selection for specific traits, such as double seeds. To create this strategy, essential parameters are: knowledge about heritability of a trait of interest combined with information about the relative cost of experimental replications and years of evaluation (Milligan et al. 1990). In this species, double seeds represent a complex trait controlled by unknown major and minor genes, affected by additive effects and by a hypothetic influence of dominance effects (Dicenta et al. 1993), with a highly variable heritability, ranging from to 0.15 (Dicenta et al. 1993) to 0.5 (Crossa-Raynaud and Grasselly 1983; Dicenta et al. 1993).

Double seeds in almond, as in other *Prunus*, come from the fertilization and development of both primary and secondary ovules, while in single seeded nuts (the most common) the secondary ovule aborts. The degeneration of the secondary ovule occurs between the pollination of the flower and the fertilization of the primary ovule (Pimienta and Polito 1982). This degeneration seems to be genetically programmed and to follow a genotype-dependent pattern (Egea and Burgos 2000). When the secondary ovule does not degenerate, a nut with two seeds will develop. Several studies observed that despite crossing parents without double seeds (or with a very low percentage), double seeded seedlings were obtained. Breeders took advantage of this situation to study the transmission of this trait

(Kester et al. 1977; Vargas et al. 1983; Crossa-Raynaud and Grasselly 1983; Dicenta et al. 1993; Sánchez-Perez et al. 2007b). In these studies, estimates of the heritability were probably biased by the selection of parents without double seeds (Kester et al. 1977; Spiegel-Roy and Kochba 1974; Dicenta et al. 1993). Furthermore, mean values of double seeds were usually higher in the offspring than in the parents (Spiegel-Roy and Kochba 1981; Vargas et al. 1983; Dicenta et al. 1993; Sánchez-Perez et al. 2007b).

Some cultivars never produce double seeds, and the cultivars that do produce double seeds show different percentages of doubles depending on the year and the geographical area in which they are grown (Grasselly and Gall 1967; Kester and Asay 1975; Spiegel-Roy 1979; Crossa-Raynaud and Grasselly 1983; Dicenta et al. 1993). Such variability is probably caused by differences in the temperatures that precede the flowering time. Based on the observations made by different authors (Grasselly 1972; Spiegel-Roy and Kochba 1974), it seems that low temperatures before blooming may increase the percentage of double seeds in almond, and so cultivars that usually produce double seeds show a higher percentage of doubles in colder areas. Likewise, Egea and Burgos (1995) found a negative relation between pre-blossom temperatures and production of double seeds and suggested that pre-blossom temperatures can influence the viability of the secondary ovule.

Another aspect associated with the abundance of double seeds could be a possible influence of the pollinator. However, González et al. (2005) suggested that the female parent mainly determined this character and Martínez-García et al. (2011) found no significant differences between pollinators for this trait.

As it has been commented above, the number of major and minor genes controlling this complex trait remains unknown, with scarce knowledge about its genetic architecture. Recently, Sánchez-Pérez et al. (2007a) mapped different traits in almond using a F1 almond population of 167 descendants from the cross between the French selection ‘R1000’ and the Spanish cultivar ‘Desmayo Largueta’. The results from the QTL analysis of doubles seeds identified a QTL (Dk-Q) in linkage group four (LG4), close to the locus UDP96-003, and explaining up to 26% of the phenotypic variance. However, dominance and additive effects were not calculated in this study.

To our knowledge a good estimation of the heritability remains needed. Therefore, the aim of this work was to study the inheritance of double seeded fruits in almond by using five

cultivars covering all the range of percentage of double seeds and their progenies, for three years.

Material and methods

Plant material

The studied trees were grown in the experimental field of CSIC in Santomera, Murcia (SE of Spain), with an irrigation system. The seedlings used in this study were obtained in 2001 by controlled pollinations between parents that usually present double seeded (DS) fruits ('Malagueña', 'Jordi' and 'Colorada') or single seeded (SS) fruits ('Ferragnès' and 'Garrigues') (Table 1). Pollen of male progenitors was collected and dried at room temperature for two days. Then it was stored at 4ºC till pollinations. Flowers at "E" stage (just before opening) of female progenitors were emasculated and hand-pollinated using a small brush. About 100 flowers were pollinated for each cross. Since the *S*-genotype of the progenitors was known (Ortega, 2002), all the crosses were cross-compatible. The selected cultivars flowered between the 10th and 24th of February. According to their parentage, the obtained seedlings were classified into four groups (DSxDS, DSxSS, SSxDS, SSxSS) (Table 2). We assumed that the selected parents are unrelated without a common ancestor, which can ensure that the inbreeding coefficient was zero (Falconer 1960).

Mature fruits were collected, the mesocarp removed and the nuts stored at room temperature for two months. Afterwards, they were stratified at 7ºC in wet vermiculite for eight weeks till germination. After germination, seedlings were taken to the field in the spring. Although the number of flowers pollinated per cross was similar, the number of seedlings obtained by family varied depending on the fruit set and germination of seeds of each cross. Four years later, when trees came into bearing, 50 mature fruits from parents and seedlings, were randomly picked up from the trees, their mesocarp removed and the nuts stored at room temperature. Each of three years of the study, the nuts were cracked using a hammer and the number of double seeds for each sample was recorded. Finally, the percentage of double seeds per genotype was calculated.

Data analysis

Two different methods were used to estimate the heritability of double seeds. Firstly, heritability was estimated by components of variance between and within families. The variance between families (Vb) and variance within families (Vw) were calculated using one-

way Anova. We used the formula H^2 = (2 x Vb)/ (Vb+Vw) to calculate the broad sense heritability (Kearsey 1965; Mather and Jinks 1982; Dicenta et al. 1993). According to Kearsey (1965), we assumed that Vb is a compound of dominance and additive measures, which makes this estimation a good approximation of the broad sense heritability (H^2).

The narrow sense heritability (h^2) was calculated by midparent-offspring regression (Falconer 1986), where the regression coefficient "b" is the heritability in narrow sense. In our experiment, where estimates of heritability are based in the same genotypes grown in the same environment, the ratio h^2/H^2 can detect the presence of dominance. Values of h^2/H^2 near one imply that the amount of non-additive variance is very small.

Differences of double seeds between groups were checked by a Bonferroni test (Bonferroni 1936). Previously, Levene's test was applied to check homogeneity of variance (Levene 1960). The values of percentage of double seeds were previously transformed by calculating the arc sine value of the square root. Finally, histograms of frequency of the percentage of double seeds in the families were obtained. In the case of unclear patterns and to check unimodality of our distributions, the R package diptest (Hartigan et al. 1985) was used.

Temperature monitoring

Temperatures were registered each hour from November to February during the three years of study and the mean values for each month were calculated using these measures.

Results

Parents performance

Table 1 shows the percentage of double seeds in the parents. As expected, 'Malagueña' 'Colorada' and 'Jordi' had double seeds, but with variability between years. 'Malagueña' was the parent with the highest value (83.3% on average). In general 'Jordi' and 'Colorada' showed similar values, with the exception of the unexpected high value observed in 2006 for 'Jordi'. 'Garrigues' and 'Ferragnès' did not show double seeds any year.

Analysis by groups

The mean values of the percentage of double seeds for each year and for the total of each family and group are showed in Table 2. Bonferroni test detected differences between years; 2006 (24.8) and 2008 (17.4) were statistically different. For year-to-year variation and for the total, Bonferroni test showed significant differences between groups. The DSxDS group presented the highest percentage of double seeds (45.1%) and the SSxSS group, the lowest (2.4%). The two groups between SS and DS presented intermediate means, but showed significant differences in 2007 (17.7% versus 3.6%) and for the total (16.8% and 7.7%).

Analysis by families

The percentage of double seeds was distributed continuously in the histogram of frequencies, ranging more or less between both parents in each family (Fig. 1). Two clear distribution patterns were observed. In the case of parents presenting high percentage of double seeds a normal distribution (Gausian) was confirmed with the results from a dip test, which were not significant for bimodal distribution (data not shown). When the female parent presented low-medium percentage of double seeds a tendency toward L-shaped distribution was more evident, especially when both parents presented null percentage of double seeds.

In most families, some individuals had lower or higher percentages than the parent with less or more doubles, respectively. In the case of 'Jordi' x 'Malagueña', some seedlings had very few double seeds (about 4%) and others overcame 'Malagueña', reaching 98% of doubles. On the other hand, although most of the descendants of 'Ferragnès' x 'Garrigues' did not produced doubles, in some years a few seedlings presented high values (around 40%).

Among the 166 seedlings analyzed, only 22 (13%) never had double seeds, many of them 11 were from the cross 'Ferragnès' x 'Garrigues', and only one from 'Colorada' x 'Malagueña' (both parents usually with double seeds) (Table 3). In contrast, in the 77 observations made during the 3 years of study in the 26 descendants of 'Jordi' x 'Malagueña', all fruit samples presented double seeds. In addition, 10.3% of the samples of 'Colorada' x 'Malagueña' showed no doubles. In the remaining families (SSxDS, DSxSS) the percentage of samples without double seeds ranged from 4.8% ('Garrigues' x 'Malagueña') to 53.3% ('Garrigues' x 'Jordi').

Heritability

The heritability of this trait calculated by both statistical methods presented a high value for each year and for the total years of study (Table 4). Our results show that the heritability by variance was higher than by regression each year. In 2007, this trait presented the highest values of heritability with 0.89 (h^2) and 1.25 (H^2). The mean value of the heritability was 0.75 (h^2) and 1.07 (H^2). Finally, the ratio h^2/H^2 ranged yearly between 0.62 and 0.75, with a mean of 0.69.

Temperature effect

Regarding monthly temperature (Table 5), the 2005-2006 period was the coolest year of the study. The lowest temperatures over the three years were measured in December 2005 (10.8 °C) and January and February 2006 (9.2 °C and 10.7 °C). For 2007 and 2008 mean temperatures were more similar and warmer. Mean of full flowering time (expressed as Julian days) of the parents was quite similar for 'Jordi' (41), 'Garrigues' (41), 'Malagueña' (44) and 'Colorada' (44) and later for 'Ferragnès' (55).

Discussion

In this study we aimed to increase the knowledge about the genetic control of the presence of double seeds in almond by studying the transmission and heritability of this trait.

Studies on the genetic control of this trait are scarce and the majority of studies come from the observation of the descendants in breeding programs, which were not specifically created to study this trait. Consequently, a general fluctuation and lack of statistical significance of the estimations was observed, mainly due to the use of parents with few or no double seeds (Dicenta et al. 1993). The specific experimental design created in this work overcomes this inconvenience.

After fertilization, the presence of double seeds is a consequence of the development of both ovules in the same ovary, and the abortion of one of the ovules is largely determined genetically. From the evolutionary point of view, the presence of both ovules in an outbred species increases the probability of success of cross-fertilization with foreign pollen. Later, the degeneration of one ovule and the development of the other without competition for reserves or nutrients, allow for optimum growth. Egea and Burgos (2000) did not find developmental stage differences at anthesis between cultivars with or without doubles. But these authors found differences three days following anthesis. They concluded that the lack of double seeds was related to disorders in secondary ovule development and indicated that the uniformity of development in the embryo sac was responsible for the presence of double seeds.

In this study, several patterns of the phenotypic distribution were observed, showing the difficulty to understand the complex genetic control of this trait. These two clear patterns, normal distribution and a tendency toward L-shaped distribution, were associated with the increment or reduction of the percentage of double seeds in the parents. On the other hand, the use of parents without double seeds (‘Ferragnès’ and ‘Garrigues’) does not seem to guarantee the absence of double-seeded descendants, since a small percentage of double seeds (2.4%) on average were observed. In this scenario, the results show that more than half of their seedlings did not produce double seeds, but some of them showed up to 38-40% of doubles in some years (Fig. 1). Dicenta et al. (1993) also studied seedlings from the cross ‘Ferragnès’ x ‘Garrigues’ (and the reciprocal) and they obtained similar results. The parents never presented double seeds and the mean percentage of double seeds in the offspring was very low (0.7 to 3.6). In addition, our results showed a transgressive segregation pattern for

this trait in nearly all families. A similar pattern of transgressive segregation was observed by Sánchez-Pérez et al. (2007b) in the 'R1000' x 'Desmayo Largueta' population.

For the first time, the use of a suitable plant material allowed us to confirm that the double seeds trait has a high heritability. Previously, Dicenta et al. (1993) observed that the use of parents with few or no double seeds affected heritability estimates for both methods. The results obtained by regression were very different each year and only significant in the first year, and the heritability calculated by variance was very low and not significant. These circumstances were also found in other almond breeding programs (Kester et al. 1977; Vargas et al. 1983). Crossa-Raynaud and Grasselly (1983) stated a strong relationship between parents and offspring for this trait. According to our precise and extended experiment, the estimation of the heritability can be considered representative for this trait, confirming that the selection of parents by their individual performance is an effective method for breeding.

In addition, according to the phenotypic distribution of the percentage of double seeds in the families studied we can classify seedlings into two groups: those that never had double seeds (abortion of secondary ovule is largely determined genetically) and those that produce doubles in varying degrees. These two groups could determine the nature of this character affected by a few loci with an additive effect and the small influence of the environment.

Similar to the majority of complex traits in plant breeding, the inheritance of this character seems to be influenced by several effects such as genotype-dependent and enviromental factors. Several hypothesis about the inheritance of this character have been proposed. From the beginning, Spiegel-Roy and Kochba (1974) stated that the inheritance of this trait was quantitative but its heritability complicated and fairly unpredictable, due to the influence of the environment. Perhaps, these results were biased by the fact that the authors used an unsuitable plant material. Spiegel-Roy and Kochba (1981) and Vargas et al. (1983) described a certain level of dominance or partial dominance, showing that seedlings had higher values than the parents. Crossa-Raynaud and Grasselly (1983) observed that when both parents presented double seeds, the percentage of seedlings with doubles in each family ranged from 22% to 100%, with a mean value between 3 to 42%. Dicenta et al. (1993), studying 2491 seedlings belonging to 51 families for three years, observed on average a higher percentage of double seeds in the offspring than in the parents, which was associated with a dominant inheritance of this trait. 'Ferragnès' x 'Garrigues' (SSxSS) was our only cross with similar

results where a possible influence of dominant effect could be considered. However, in our study no deep information can be addressed about the nature of the genetic component affecting the variation of this trait.

According to our results this trait seems to be controlled by several minor QTLs, with the presence of several genes with small effects. Sánchez et al. (2007a) used a mapping population from the cross between 'R1000' and 'Desmayo Largueta' to identify a QTL for double kernel (Dk-Q) in the linkage group four (LG4), explaining up to 26.4% of the phenotypic variance. However, no other QTLs associated with this trait were identified, maybe due to the fact that both parents presented low or null percentage of double seeds, hindering the understanding of this complex trait.

Egea and Burgos (1995) observed a strong negative correlation between temperatures during pre-blossom months (especially in December r =- 0.78) and the percentage of doubles, in eight almond cultivars. These results suggested that lower temperatures (9-10ºC) during this period favored the presence of double seeds, while the higher temperatures (12-13ºC) induced a faster development of the primary ovule preventing the development of the secondary ovule, but would not affect fruit set. The important effect of temperature on the expression of this character is generally accepted (Grasselly and Gall 1967; Kester and Asay 1975; Spiegel-Roy 1979; Crossa-Raynaud and Grasselly 1983) with low temperatures before flowering increasing double seeds (Grasselly 1972; Spiegel-Roy and Kochba 1974). According to this theory, cultivars that produce few double seeds in warm areas are expected to increase this value in colder ones. In our study, the influence of low temperature, previous to the flowering time on double seeds was also shown. These results confirm the genotype-by-environment interaction effect affecting to this trait. In fact, 2005-2006 presented the lowest temperature in December (10.8), January (9.2) and February (10.7), and both parents and descendants had the highest percentages of double seeds throughout the three year study.

Conclusions

Our results accurately show the high heritability of double seeds and an influence of low temperatures prior to blooming time, on this trait. A deeper study to obtain the missing genetic variation and the possible candidate genes associated with this trait is needed to build a new marker-assisted breeding strategy. Until then, a clever solution to prevent the presence

of double seeds in the offspring in the breeding programs is to avoid as much as possible the use of double seeded parents.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1 Double seeds (%) in the parents for the three years of study in a sample of 50 fruits.

Table 2 Crosses, number of seedlings analysed (n) and average values for the percentage of double seeds in the parents (X) and in the offspring (Y), for each year and for all years as a whole. Different vertical letters represent significant differences between groups, and different horizontal letters represent significant differences between years.

Table 3 Samples (%) without double seeds by families and by years. Seedlings (%) that never presented double seeds.

Table 4 Inheritance of double seeds by regression and variance components for each year and for all years as a whole.

Table 5 Monthly mean temperatures (ºC) before and during flowering time for the three years of study.

Fig. 1 Percentage of seedlings with double seeds in each family.

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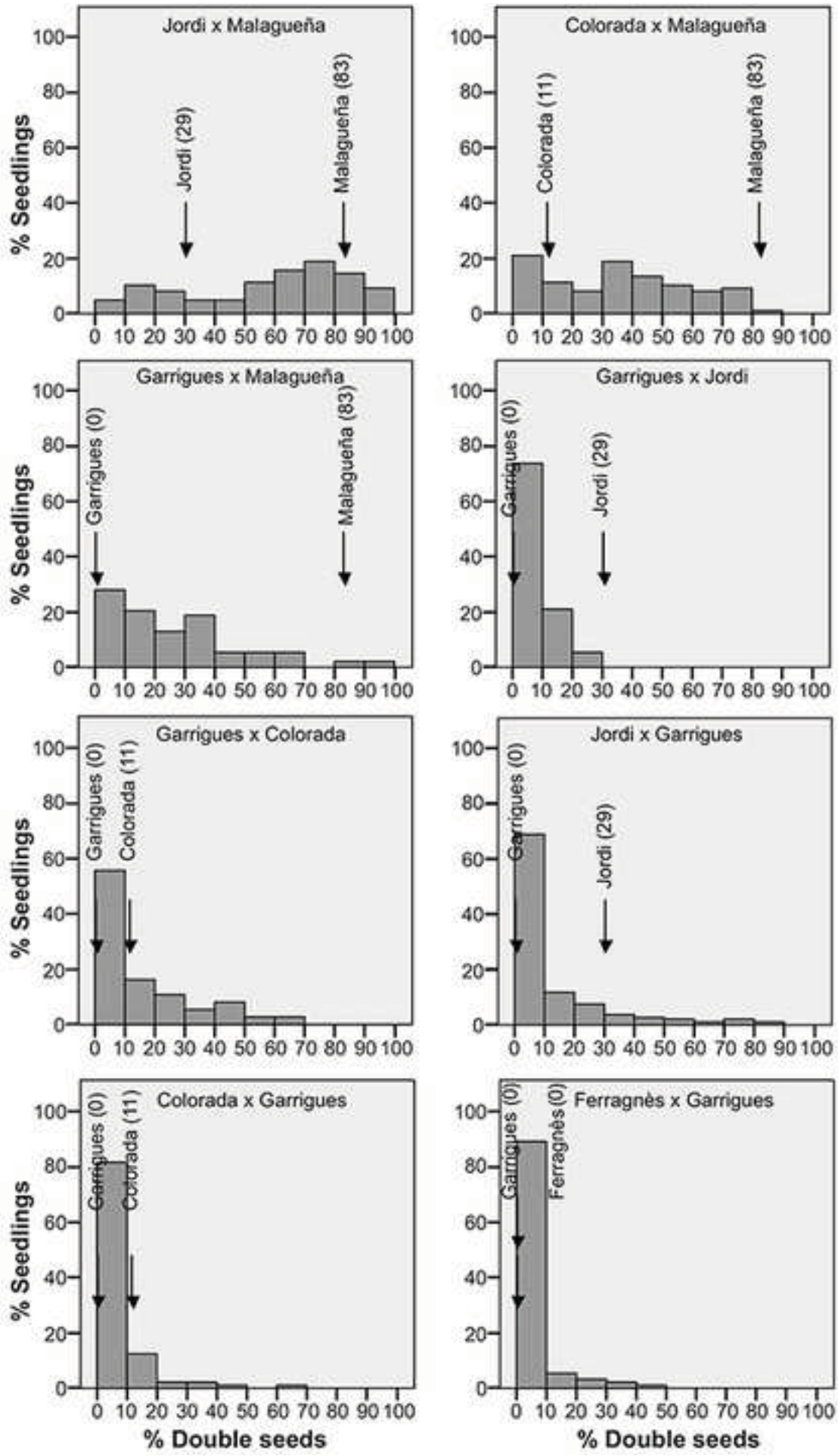

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Table 1 Double seeds (%) in the parents for the three years of study in a sample of 50 fruits.

Cultivar	*S*-genotype	Origin	2006	2007	2008	Mean
Malagueña	$S_{22}S_{23}$	Spain	84.0	80.0	86.0	83.3
Jordi	S_5S_6	Spain	66.0	14.0	8.0	29.3
Colorada	$S_{12}S_{28}$	Spain	8.0	18.0	6.0	10.6
Garrigues	$S_{13}S_{27}$	Spain	0.0	0.0	0.0	0.0
Ferragnès	S_1S_3	France	0.0	0.0	0.0	0.0

Table 2 Crosses, number of seedlings analysed (n) and average values for the percentage of double seeds in the parents (X) and in the offspring (Y), for each year and for all years as a whole. Different vertical letters represent significant differences between groups, and different horizontal letters represent significant differences between years.

		2006		2007		2008		Mean	
	n	X	Y	X	Y	X	Y	X	Y
Jordi x Malagueña	26	75.0	59.1	47.0	53.1	47.0	52.6	51.0	55.0
Colorada x Malagueña	26	46.0	36.5	49.0	36.3	46.0	33.0	47.3	35.3
Mean DSxDS*		60.5	47.8a	48.0	44.7a	46.5	42.6a	49.1	45.1a
Garrigues x Malagueña	14	42.0	30.8	40.0	26.9	43.0	17.6	41.6	25.1
Garrigues x Jordi	5	33.0	5.4	7.0	2.4	4.0	1.2	9.4	3.0
Garrigues x Colorada	11	4.0	16.3	9.0	12.9	3.0	8.0	5.7	12.4
Mean SSxDS*		26.3	21.2b	18.6	17.7b	16.6	11.3b	18.9	16.8b
Jordi x Garrigues	29	33.0	20.1	7.0	3.2	4.0	5.7	9.4	9.6
Colorada x Garrigues	27	4.0	8.5	9.0	3.9	3.0	4.6	5.7	5.6
Mean DSxSS*		18.5	14.5b	8.0	3.6c	3.5	5.2b	7.6	7.7c
Ferragnès x Garrigues	28	0.0	5.5	0.0	0.4	0.0	1.1	0.0	2.4
Mean SSxSS*		0.0	5.5c	0.0	0.4d	0.0	1.1c	0.0	2.4d
Mean Year			24.8a		18.6ab		17.4b		

*Abbreviation: SS, single seeded. DS, double seeded

Table 3 Samples (%) without double seeds by families and by years. Seedlings (%) that never presented double seeds.

		Samples				Seedlings (%)
	N	2006	2007	2008	Mean	
Jordi x Malagueña	26	0.0	0.0	0.0	0.0	0.0
Colorada x Malagueña	26	15.4	7.7	7.7	10.3	3.8
Mean DSxDS		7.7	3.8	4.0	5.1	1.9
Garrigues x Malagueña	14	0.0	0.0	14.3	4.8	0.0
Garrigues x Jordi	5	40.0	60.0	60.0	53.3	0.0
Garrigues x Colorada	11	36.4	36.4	36.4	36.4	18.2
Mean SSxDS		25.3	32.1	36.9	31.5	6.1
Jordi x Garrigues	29	32.1	37.9	41.4	37.2	10.3
Colorada x Garrigues	27	34.6	55.6	53.8	48.0	18.5
Mean DSxSS		33.4	46.7	47.6	42.6	14.4
Ferragnès x Garrigues	28	60.7	85.2	84.6	76.8	39.3
Mean SSxSS		60.7	85.2	84.6	76.8	39.3
Mean		31.8	42.0	43.2	39.0	

Table 4 Inheritance of double seeds by regression and variance components for each year and for all years as a whole.

By regression				By variance			
2006	2007	2008	Total	2006	2007	2008	Total
0.63±0.14	0.89±0.13	0.73±0.16	0.75±0.08	0.84	1.25	1.17	1.07

Table 5 Monthly mean temperatures (ºC) before and during flowering time for the three years of study.

	2005-2006	2006-2007	2007-2008	Mean
November	13.8	13.8	13.4	13.7
December	10.8	11.6	12.1	11.5
January	9.2	11.8	12.3	11.1
February	10.7	14.1	12.6	12.5
Total	11.1	12.8	12.6	12.2