Cross-compatibility of the Parents as the Main Factor for Successful Olive Breeding Crosses

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ABSTRACT. Previous analyses of olive (*Olea europaea* L.) crosses have revealed an important degree of pollen contamination. For this reason, the authors tested the paternity of a set of progenies coming from crosses among different cultivars within the olive breeding program of Córdoba, Spain, using four polymorphic microsatellites. They found that the expected pollen sired 141 (83.4%) of the 169 samples analyzed. The contamination rate was either almost total or almost null within each particular cross considered, not being comparable between different crosses. In a second experiment the authors evaluated the influence of several factors on the success of olive crosses, the type of isolation bag, the timing and number of pollinations, and the cross-compatibility of the parents in a multifactorial assay in 2003. They observed no differences in the type of pollination bag used or the number and timing of pollen additions when they analyzed 145 seeds. The main factor affecting the success of the crosses seems to be the intercompatibility among cultivars, because it had a significant influence on the rate of contamination. In the failed crosses, the authors clearly detected the contribution of more than one cultivar to the paternity. The results obtained here indicate that some knowledge of cross-compatibility relationships in olive is required to design crosses in olive breeding programs effectively. To achieve this objective, the progeny parentage analysis could be of great help because of the high level of pollen contamination found among those studied here.

Growers have obtained most of the current olive cultivars by empiric selection over centuries (León et al., 2005). Several breeding programs are now devoted to releasing new cultivars better adapted to the actual trends in olive growing in different Mediterranean countries, such as Israel (Lavee, 1990), Italy (Bellini, 1993; Fontanazza and Baldoni, 1990), or Spain (Rallo, 1995).

Having a reliable cross method is one of the first essential steps for the progress of any breeding program. In this sense, those crosses whose progeny have been actually fertilized by the expected pollen donor (the cultivar used as the father during the crosses) are successful. Olive is a wind-pollinated species in which adding pollen to bagged branches of the maternal parent has been the usual cross protocol in breeding programs (Bellini, 1993; Fontanazza and Baldoni, 1990; Lavee, 1990; Rallo, 1995). Several authors have tried cloth and paper bags in previous experiments, obtaining higher fruit set in those branches isolated with the latter (Griggs et al., 1975). However, in all cases, they have only measured the

pollination effectiveness of the breeding crosses by the fruit set obtained, without considering that this can be the result of fertilization by different cultivars. So, we consider necessary the use of molecular markers in paternity tests to evaluate the success of the pollinations performed.

Different researchers have undertaken similar methodologies based on the measure of the fruit set to determine adequate pollen donors of certain olive cultivars (Morettini et al., 1972; Moutier, 2002), and in some cases they have complemented the studies with pollen tube growth tests (Cuevas and Polito, 1997; Wu et al., 2002). Nevertheless, the results were not always consistent (Lavee et al., 2002), because the data coming from different years allow one to classify the same pair of cultivars as cross-compatible and cross-incompatible.

Simple sequence repeats (SSRs), or microsatellites, are increasingly becoming the markers of choice for paternity analysis in plants, because they now offer an easy and reliable way to test the paternity of seeds and seedlings by checking the presence of parental alleles in the descendants (Morand-Prieur et al., 2003; Nishizawa et al., 2005). Their codominant inheritance and high polymorphism make it possible to obtain high combined parentage exclusion probability values with a relatively low number of hypervariable loci (Díaz et al., 2006; Isagi et al., 2004). Several authors have used this technique in olive to examine the paternity of seedlings (De la Rosa et al., 2004) and even of ungerminated seeds (Díaz et al., 2006; Mookerjee et al., 2005). In fact, some of these studies have revealed a high occurrence of contamination with undesirable pollen among the progeny of both controlled self- and cross-pollinations in olive (De la Rosa et al., 2004; Díaz et al.,

2006). Furthermore, those findings have avoided coming to wrong conclusions about the male parent effect on the length of the juvenile period (Santos-Antunes et al., 2005) and on several agronomic characteristics (León et al., 2004a, b) in studies performed in the olive breeding program of Córdoba, Spain, because they have revealed that in some cases the real paternity is not the expected one.

The purpose of this research was to determine the impact of the type of pollination bag, the number and timing of pollen applications, and the cultivars used as parents on fertilization success in olive. For this, we tested the paternity of olive seeds using microsatellite markers.

Materials and Methods

PATERNITY TESTING IN THE ROUTINE BREEDING PROGRAM OF CÓRDOBA. We tested the paternity of the offspring of eight olive crosses made using six different cultivars from the breeding program of Córdoba (Table 1). We made the crosses in two different locations in southern Spain: at the World Olive Germplasm Bank of the Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica (IFAPA) "Alameda del Obispo" (Córdoba) during 2000 and 2002, and at the cultivar collection of IFAPA "Venta del Llano" (Mengíbar, Jaén) in 2001.

We carried out the crosses using the two types of bags described later. We collected the pollen from paternal trees located in the World Olive Germplasm Bank and stored it at –20 °C until its utilization. We determined pollen viability before use by optical microscopy, using acetic carmine and also by observing the pollen tube length after its germination during 24 to 48 h at 25 °C in a solution consisting of 10% sucrose, 0.01% boric acid, and 10 mg·L^{-1} tetracycline (Fernández-Escobar et al., 1983). We mixed the pollen with six parts of talc to increase its volume and then we sprayed it three times every 2 d onto the maternal bagged branches, as done in previous experiments (De la Rosa et al., 2004; Díaz, 2005). In all cases, we performed two replicates, choosing two maternal trees per cultivar in each cross.

STUDY OF FACTORS AFFECTING CROSS-POLLINATION SUCCESS. To test the influence of different factors on the success of olive crosses, we performed a pollination assay in the World Olive Germplasm Bank during 2003. We bag isolated the flowering branches of the maternal tree well before anthesis [with buds in DII phenological stage: the corolla size becoming larger than the calyx size (Tous and Romero, 1993)] to avoid any contact with airborne pollen. The bagged shoots bore 144 inflorescences on average. We devised a multifactorial design for studying the effect of the following three factors on the same bagged shoot (Table 2) and, as done previously, two replicates (in two different mother trees of the same cultivar) were made:

1. *Pollination bag material.* We investigated the influence of two types of branch isolation bagging material: double-plastic perforated (1-mm-diameter holes) and Tyvek (DuPont, Wilmington, DE) (Del Río and Caballero, 1999; Smith and Mehlenbacher, 1994). Researchers have previously used both of them in olive controlled pollinations within the aforementioned breeding program in Córdoba.
2. *Number and timing of pollen additions.* We carried out three different pollination methodologies: a single pollination just before anthesis (P1); three pollinations, one every 2 d, starting

Table 1. Pollen contamination detected in olive crosses carried out from 2000 to 2002 in Córdoba and Jaén (Andalusia, Spain).

Year	Location	Cross[z]	Analyzed seeds (n)	Contaminated seeds (n)	Putative father (no. of seeds)[z,y]	Paternal alleles (n)			
						ssrOeUA-DCA3	ssrOeUA-DCA9	ssrOeUA-DCA18	EMO3
2000	Córdoba	P × LS	11	0	LS (11)	2	2	2	2
		LS × P	18	0	P (18)	2	2	2	2
		P × A	15	0	A (15)	2	2	2	1[x]
2001	Jaén	P × A	20	0	A (20)	2	2	2	1[x]
		MS × A	45	0	A (45)	2	2	2	1[x]
		A × Me	20	20	P (10), H (1), U (9)	5	6	5	4
		A × K	10	8	P (4–6), LS (1–3)[w], K (2), U (1)	3	4	3	4
2002	Córdoba	A × MS	10	1	MS (9), P (1)	3	3	3	2
		MS × K	10	0	K (10)	1[v]	2	1[v]	2
		MS × A	10	0	A (10)	2	2	2	1[x]

[z]A, 'Arbequina'; H, 'Hojiblanca'; K, 'Koroneiki'; LS, 'Lechín de Sevilla'; Me, 'Memecik'; P, 'Picual'; MS, 'Manzanilla de Sevilla'; U = unknown.
[y]No selfing products found, suggesting the possible self-incompatibility of some of these cultivars, as previously reported in the case of 'Arbequina' (Díaz et al., 2006) and 'Picual' (De la Rosa et al., 2004; Díaz et al., 2006).
[x]'Arbequina' is homozygous for the microsatellite EMO3.
[w]'Lechín de Sevilla' and 'Picual' share a common allele at three of the four simple sequence repeat loci amplified, and one allele of the remaining locus matches in both of them with the maternal ones, making it impossible to assign a single father to two of the seeds analyzed.
[v]'Koroneiki' olive is homozygous for the microsatellites ssrOeUA-DCA3 and ssrOeUA-DCA18.
The total number of seeds analyzed, the number of contaminated seeds, the most probable father of every seed, and the number of paternal alleles identified per cross are indicated.

Table 2. Pollen contamination of olive crosses, carried out in 2003, as influenced by the cross (female × male parent), bagging material (plastic or Tyvek), and pollination treatment [only one pollination just before anthesis (P1); three pollinations, one every 2 d, starting just before anthesis (P2); three pollinations, one every 2 d, starting 3 d after anthesis (P3)].

| | Analyzed seeds (n) | | | | | | | | | | |
| | P1 | | | P2 | | | P3 | | | | |
Cross[z]	Plastic	Tyvek	Total	Plastic	Tyvek	Total	Plastic	Tyvek	Total	Per cross (N)	Putative father (no. of seeds)[z,y]
A × K	10 (10)	—	10 (10)	7 (7)	3 (3)	10 (10)	5 (4)	5 (5)	10 (9)	30 (29)	P (3), GS (2), LS (2), K (1), MS (1), U (21)
A × MS	5 (2)	5 (0)	10 (2)	4 (0)	6 (0)	10 (0)	5 (0)	5 (0)	10 (0)	30 (2)	MS (26), GS (2), md (2)
K × A	5 (4)	5 (5)	10 (9)	6 (5)	4 (4)	10 (9)	5 (4)	5 (5)	10 (9)	30 (27)	H (9), A (3), P (3), MS (1), U (14)
K × MS	6 (0)	4 (1)	10 (1)	5 (0)	5 (0)	10 (0)	5 (0)	5 (0)	10 (0)	30 (1)	MS (29), U (1)
F × K	5 (4)	—	5 (4)	10 (10)	—	10 (10)	5 (5)	5 (3)	10 (8)	25 (22)	P (7), H (2), K (2), A (1), LS (1), MS (1), U (10), md (1)

[z]A, 'Arbequina'; F, 'Frantoio'; GS, 'Gordal Sevillana'; H, 'Hojiblanca'; K, 'Koroneiki'; LS, 'Lechín de Sevilla'; md, missing data; MS, 'Manzanilla de Sevilla'; P, 'Picual'; U, unknown.
[y]No selfing products found, suggesting the possible self-incompatibility of some of these cultivars, as previously reported in the case of 'Arbequina' olive (Díaz et al., 2006).
For each case, the total number of seeds analyzed and the number of contaminated seeds (in parentheses) are given. The total number of analyzed seeds and the contaminated ones found (in parentheses) per cross, as well as the most probable father of every seed are also indicated.

just before anthesis (P2); and three pollinations, one every 2 d, starting 3 d after anthesis (P3).

3. *Cross-compatibility between cultivars.* We carried out five different cross combinations of 'Arbequina', 'Frantoio', 'Koroneiki', and 'Manzanilla de Sevilla' olive. We were interested in testing one cross that had previously worked well ('Arbequina' × 'Manzanilla de Sevilla'), and another in which we had detected a high pollen contamination rate ('Arbequina' × 'Koroneiki'). Additionally, all these cultivars show very interesting characteristics from a breeding point of view.

We selected a total of 30 seeds per cross for the paternity tests, 10 per treatment, five from every one of the two types of bags (Table 2), when available.

DNA ISOLATION FROM OLIVE OFFSPRING AND MICROSATELLITE AMPLIFICATION. We used two different DNA extraction approaches. For the progenies from the Córdoba routine breeding program, we germinated the seeds and isolated the DNA from leaves of the olive seedlings according to Murray and Thompson (1980) with slight modifications (De la Rosa et al., 2002). In the pollination experiment, we extracted the DNA directly from uncoated seeds as described by Díaz et al. (2006). We performed the DNA extractions using the DNeasy Plant Mini kit (Qiagen, Valencia, CA) and followed the supplier's instructions. We collected a total of 314 samples (one seed per fruit) for DNA isolation and amplification (Tables 1 and 2).

We chose a set of four olive microsatellites with great informative potential (high polymorphism) and proved amplification efficiency (De la Rosa et al., 2004; Díaz et al., 2006)—ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA18 (Sefc et al., 2000), and EMO3 (De la Rosa et al., 2002)—for the paternity analyses.

We amplified the DNA by polymerase chain reaction (PCR) in 15-μL volume solutions with 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each deoxyribonucleotide triphosphate (dNTP), 0.2 μM of forward (fluorescently labeled) and reverse primers, 1 U Ampli*Taq*-Gold polymerase (Applied Biosystems, Foster City, CA), and 15 ng genomic DNA template. We performed all the reactions in a Gene Amp PCR system 9600 (Applied Biosystems) under the following profile: 11-min initial denaturation step at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at the annealing temperature reported by the previous authors (De la Rosa et al., 2002; Sefc et al., 2000), 2 min at 72 °C, with a final extension step of 7 min at 72 °C. We diluted the amplicons 5- to 20-fold before analysis on an ABI 310 (Applied Biosystems) automatic sequencer running GeneScan software (version 3.7; Applied Biosystems).

We always confirmed the maternal allelic contribution and, when pollen contamination was detected, we inferred the putative male parent of each progeny by removing the maternal alleles from the complete genotype at the four microsatellites used, thanks to the availability of previous genotyping data (De la Rosa et al., 2004; Díaz, 2005; Rallo, 2001). The paternity assignment was made by eye and only when all paternal alleles for the four markers matched the putative father.

Results and Discussion

CONTROLLED CROSS-POLLINATION WITHIN A ROUTINE BREEDING PROGRAM. We were able to discriminate between seeds produced by the hand pollination performed and those coming from the fertilization by undesirable pollen thanks

to the high combined parentage exclusion probability value (0.92) provided by the four microsatellites used here and in a previous study (Díaz et al., 2006). We analyzed the paternity of 169 seedlings coming from eight different crosses performed in 3 different years within the Córdoba breeding program (Table 1). We found that the expected pollen sired 141 (83.4%) of the 169 samples analyzed. The contamination rate was not comparable between all crosses. Quite the opposite, the contamination was either almost total or almost null within every cross (Table 1). We confirmed the expected parents for seedlings coming from the crosses 'Manzanilla de Sevilla' × 'Arbequina', 'Picual' × 'Lechín de Sevilla' and their reciprocal ones, and 'Picual' × 'Arbequina' and 'Manzanilla de Sevilla' × 'Koroneiki'. In the case of the crosses 'Arbequina' × 'Koroneiki' and 'Arbequina' × 'Memeçik', the level of contamination with foreign pollen was nearly total. We could identify 'Picual' olive as being the primary pollen donor in these contaminated crosses, therefore confirming previously reported results (De la Rosa et al., 2004; Díaz et al., 2006). We could not determine the pollen donor in some cases (5.9%). This is not unusual because the genotypic profile for the four SSRs used here was not available for many cultivars grown in the two previously mentioned germplasm collections. In light of these results, we designed an experiment aimed at figuring out the determining factors for obtaining pure offspring from olive crosses.

STUDY OF FACTORS AFFECTING CROSS-POLLINATION SUCCESS. Pollen from a different source to that added to the bagged shoots sired a total of 81 (55.9%) of 145 seeds analyzed in this experiment (Table 2). As noted previously, we could not determine the pollen donor in some cases (22.1%), although the paternal alleles identified in those seeds (more than two per marker) clearly suggest the contribution of more than one cultivar to their paternity. The distribution of those contaminated seeds among crosses is indicative of the main factor affecting the success of the fertilizations, as shown here:

1. *Pollination bag material*. Although we expected the amount of pollen able to gain access to bagged flowers to be higher when we used perforated plastic bags, neither the contamination ratio with undesirable pollen within each cross (Table 2) nor the fruit set measured inside both types of bags (data not shown) were related to the type of bag used in pollination tests. So, the paternity analyses have demonstrated the permeability of both materials to airborne pollen. Interestingly, this lack of isolation did not hamper successful fertilizations in certain cultivar combinations. Transparent plastic bags offer the possibility of monitoring floral and fruit development and, therefore, should be selected if this information is considered of interest.

2. *Number and timing of the pollen additions*. We observed no differences in the contamination rate between the fruit obtained in the three treatments carried out during 2003 (Table 2), although in the first two (P1 and P2) we started pollination just before anthesis, and in the last one (P3) we started pollination 3 d after anthesis. Similarly, we have not detected any possible influence of the number of pollinations, one in treatment P1 and three in the remaining treatments P2 and P3, on the effectiveness of cross methods. This indicates that only one pollination just before anthesis could be sufficient to have a pure progeny. Because pollen is quite laborious to collect and it is obtained in small quantities, protocol P1 could represent important time and effort savings.

3. *Cross-compatibility between cultivars*. We observed noticeable differences in fertilization success by the pollen supplied. The high degree of contamination of some crosses and the purity of others indicate a strong influence of the cultivar combination on the effectiveness of the pollinations independent of the pollination bag material used or the number and timing of the pollen additions (Table 2). Interestingly, two of the combinations with the highest levels of contamination with undesirable pollen are reciprocal ('Arbequina' × 'Koroneiki' and vice versa). De la Rosa et al. (2004) obtained similar results for the cultivar combination 'Arbequina'–'Zaity' in previous experiments. These results are consistent with those obtained in the preliminary assays (Table 1). As in that case, we identified predominant pollen donors when we used particular cultivars as the maternal parent.

A multifactorial analysis of variance revealed that cross-compatibility between cultivars was the only factor showing a significant influence on the rate of contamination ($F = 67.58$, $P < 0.001$). According to these results, intercompatibility between cultivars acting as parents seems to be the most important factor among those studied to achieve success in obtaining desired progeny. The type of bag, and the timing and number of pollinations do not seem to be crucial factors. Furthermore, we did not observe spontaneous self-pollinations, even when we carried out the pollen additions after anthesis, which is in agreement with a previous study about self-incompatibility in olive (Díaz et al., 2006).

CROSS-COMPATIBILITY RELATIONSHIPS. The cultivars considered here seem to exhibit reciprocal intercompatibility relationships; that is, a certain cultivar pair behaves in the same way independently of which cultivar acts as the male or the female parent (Table 3). This suggests that a gametophytic incompatibility system might be acting in olive (Mookerjee et al., 2005; Sedgley, 1994). In fact, olive flowers have a wet stigma (Ciampolini et al., 1983) and generally binucleate pollen (Bradley and Griggs, 1963), which are considered to be two signs of gametophytic self-incompatibility (Sedgley, 1994).

Table 3. Compatibility relationships [cross-compatible (c), cross-incompatible (i)] between the olive cultivars used in the present work, as revealed by paternity analysis.

Female	Male						
	Arbequina	Frantoio	Koroneiki	Lechín de Sevilla	Manzanilla de Sevilla	Memeçik	Picual
Arbequina			i		c	i	
Frantoio			i				
Koroneiki	i				c		
Lechín de Sevilla							c
Manzanilla de Sevilla	c		c				
Memeçik							
Picual	c			c			

In this sense, we always obtained pure progenies from crosses 'Picual' × 'Lechín de Sevilla', 'Manzanilla de Sevilla' × 'Arbequina' and 'Manzanilla de Sevilla' × 'Koroneiki', and the reciprocal ones. On the contrary, we found that the contamination was almost total when we tested the progeny from the cross 'Arbequina' × 'Koroneiki' and the reciprocal one. Additionally, we can classify the pair 'Picual' × 'Arbequina' olive as cross-compatible, whereas 'Arbequina' × 'Memeçik' and 'Frantoio' × 'Koroneiki' olive are cross-incompatible (data corresponding to the reciprocal crosses are not available).

The cultivar associations obtained (Table 3) are in accordance with previous self- and cross-pollination results (De la Rosa et al., 2004; Díaz, 2005; Díaz et al., 2006) and with the predominant pollen donors identified in contaminated crosses here and in previous studies. As an example, 'Manzanilla de Sevilla' was one of the main sources of contaminant pollen when 'Arbequina' was used as the mother in self-pollination tests. So, the good results derived from the cross 'Arbequina' × 'Manzanilla de Sevilla' are not surprising (Tables 1 and 2). This contradicts a previous report made when testing only the fruit set, in which the author considered 'Arbequina' olive as a bad pollinator of 'Manzanilla de Sevilla' olive (Moutier, 2002). Similarly, 'Arbequina' olive appears as one of the most frequent pollen donors in 'Picual' tree self-pollinations in the previous studies (De la Rosa et al., 2004; Díaz et al., 2006) and, consequently, the cross 'Picual' × 'Arbequina' also worked correctly (Table 1).

The cross-compatibility information (Table 3) could also be useful in those cases in which growers require the presence of pollen donors (Moorkerjee et al., 2005), such as in areas where they are replacing the existing diversity of cultivars by an increasing homogeneity of high productive ones (Junta de Andalucía, 2002) or in countries where olive is not a native tree and they have imported a few cultivars from traditional olive-growing areas. However, in the current study, we established the cross-compatibility relationships between cultivars under artificial conditions (controlled pollinations), so breeders and growers must verify the overlapping of both parental cultivar bloom periods in particular locations and environments to guarantee cross-compatibility in situ. According to the bloom periods reported by Barranco and Rallo (2005) in research carried out during 6 years in Córdoba (Spain), the cultivar combinations 'Arbequina'–'Manzanilla de Sevilla' and 'Picual'–'Lechín de Sevilla' (in both reciprocal crosses) and 'Picual' × 'Arbequina' should also be compatible in field conditions because their blooms occur simultaneously (data on 'Koroneiki' olive bloom period are not available).

The average temperature and relative humidity during the blooming period (April and May) in the locations in which we carried out the crosses were 17.5 °C and 67.3% in 2000, 17.0 °C and 60.1% in 2001, 16.7 °C and 64.2% in 2002, and 18.6 °C and 59.5% in 2003 respectively. Even though the climatic conditions are supposed to affect the compatibility behavior of the olive cultivars (Bradley and Griggs, 1963), we have obtained consistent results in all the crosses made in two different years ('Picual' × 'Arbequina', 'Manzanilla de Sevilla' × 'Arbequina', 'Arbequina' × 'Koroneiki', and 'Arbequina' × 'Manzanilla de Sevilla'; Tables 1 and 2). These results agree with those obtained in a previous study in which the self-incompatibility of 'Arbequina' and 'Picual' olive revealed invariable in different environments (Díaz et al., 2006).

In short, cross-compatibility relationships are revealed as being a vital factor in the success of olive crosses and, therefore, of breeding programs. This information is also of great interest in the design of new plantations. Microsatellite markers could be very useful to confirm future results about the genetic control of the compatibility system acting in olive, which is still unknown.

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