Open-pollination Provides Sufficient Levels of Cross-pollen in Spanish Monovarietal Olive Orchards

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Additional index words. *Olea europaea*, self-incompatibility, pollination deficits, pollen dispersal, seedling paternity

Abstract. Most Spanish olive orchards are monovarietal as a result of the farmer's belief that the species does not require cross-pollination. Paradoxically, accumulated evidence from controlled experiments demonstrates that olive is partially self-incompatible and that cross-pollination increases yield and fruit quality in this wind-pollinated crop. With the aim of assessing cross-pollination deficit in large plots of the most widespread olive oil cultivar in Spain, fruit set was compared in two solid orchards of 'Picual' in response to self-, open-, and artificial cross-pollination. In both orchards, 'Picual' behaved as a self-incompatible cultivar with reduced fruit set under self-pollination (index of self-incompatibility = 0.21). However, cross-pollination rarely increased fruit set in comparison with open-pollination. Bagging experiments demonstrated that open-pollination provided enough cross-pollen to induce high levels of fruit set. The increase in fruit set in response to mechanical application of cross-pollen was limited to the trees directly receiving the pollen flow and only in one orchard. Consistently, airborne pollen concentration after a single terrestrial application significantly fits a decay curve with a short dispersal. In contrast with the limited dispersal of single mechanical applications, open-pollination results suggest that trees from plantations at least 250 to 500 m away are acting as unsuspected pollenizers. This is probably taking place to some extent in most traditional olive districts in Spain, and it explains why farmers have never demanded pollination designs in this crop. Modern homogeneous plantations can, however, change this situation dramatically and give rise to pollination deficits.

In Spain, olive has been cultivated since antiquity in large, supposedly monovarietal masses without pollination design and without farmers noticing fruit set deficiencies. However, accumulated evidence from carefully designed experiments shows that olive is partially self-incompatible (Cuevas et al., 2001; Lavee and Datt, 1978; Lavee et al., 2002; Sibbett et al., 1992). Olive is wind-pollinated. Isolated 'Manzanillo' plantations in the United States and Israel have shown cross-pollination deficits that can be solved by regrafting some trees with suitable pollenizers (Lavee and Datt, 1978) or by mechanical application of compatible pollen (Sibbett et al., 1992). Some authors have proposed 30 to 40 m as the maximum distance from pollenizers (Griggs et al., 1975; Lavee and Datt, 1978).

This dependence on cross-pollination is not reflected in most olive regions in Spain, where even modern plantations do not incorporate pollination designs. To find out the

reasons for this apparent contradiction, we carried out a study to determine whether cross-pollination deficits exist in the most widespread olive cultivar in Spain by comparing fruit set in response to self-, open-, and cross-pollination (mechanically applied). Pollen dispersal studies and seed paternity analyses were performed to confirm pollination success.

Materials and Methods

Cross-pollination deficit assessment. The experiments were carried out in two monovarietal orchards of 8-year-old 'Picual' trees located in Sorbas (37°09' N, 2°09' W) and Tabernas (37°05' N, 2°18' W) (Almería, Spain) in 2000 and 2001, respectively. In Sorbas, trees of different cultivars (Hojiblanca, Arbequina, and Manzanillo) were 250 m away. In Tabernas, the closest possible pollenizers were 500 m away and were of cultivars Hojiblanca, Arbequina, and Lechín de Granada.

Nine rows of trees were used for cross-pollination deficit assessment. Four contiguous heavy-flowering trees of 'Picual' were selected in Rows 1, 2, 3, 5, and 9. Because orchard spacing was 6 × 6 m in Sorbas and 7 × 7 m in Tabernas, Row 9 was 48 or 56 m away, respectively, from the first row of trees. Final fruit set was measured 45 d after bloom in 10 shoots per treatment in each of these trees and

expressed as the number of fruits per panicle. Tagged shoots were hand-thinned before bloom at the same load of 12 panicles per shoot. Self-pollination was achieved by bagging shoots individually using tissue paper sacks before anthesis. Open-pollination consisted of the free exposition of the shoots to wind-pollination. Cross-pollination was performed by applying viable cross-pollen from a distance of 3 m directly to the four experimental trees of the first row. Pollen was applied with a hand-operated duster (Model 150DX; Maruyama, Mfg. Co. Inc., Tokyo, Japan). Pollen application consisted of 60 cm³ of a mixture of olive pollen and a dilutor and it was carried out three times in 2000 and twice in 2001. 'Arbequina' and 'Hojiblanca' pollen were diluted to 50% with cypress pollen in 2000 and to 25% with *Lycopodium* spores in 2001. 'Arbequina' and 'Hojiblanca' compatibility with 'Picual' was known from previous research (Cuevas et al., 2001). Open-pollinated shoots were bagged during cross-pollen application and remained so for a further 24 h. An extra cross-pollination treatment was added in which the shoots were bagged all the time except during the mechanical application of cross-pollen. This treatment was implemented only in the first row to control the negative effects of bagging. The index of self-incompatibility (ISI), defined as the ratio of fruit set after self-pollination to that following cross-pollination (Zapata and Arroyo, 1978), was calculated. The experimental design was a randomized complete block in which each tree acts as a block and replication. Each tree row was analyzed separately. Fruit set data were analyzed by analysis of variance and means were compared by Tukey's test ($P < 0.05$) using Statistix 8.0 (Analytical Software, Tallahassee, FL).

Pollen dispersal. To determine the effective pollination distance for artificial pollination using a hand-duster, we designed two experiments. The first one measured olive pollen concentration at increasing distances from the application point; the second experiment counted the number of pollen grains captured by olive flowers of trees at increasing distances from the application point.

The first experiment was carried out in an open field on a calm November day when airborne olive pollen was negligible (in Northern Hemisphere olive blooms typically in May). To model pollen dispersal after hand-duster application at 150 cm height, we placed at ground level glass slides (25 × 40 mm) coated with a gentle adhesive at 1, 2, 4, 8, 16, 32, and 100 m (one slide per distance) from the point where olive pollen was applied (60 cm³). The number of pollen grains in 8 cm² was counted and represented as a function of the distance. The model of pollen dispersal best fitting the results was determined using Statgraphic Plus 4.0 (Manugistic, Scottsdale, AZ).

In the second experiment, 60 cm³ of a 1:1 mixture of olive and cypress pollen (acting as a marker of olive pollen density on stigmas) was applied to a single olive tree during the 2002 blooming period. To calculate the

Received for publication 7 Oct. 2008. Accepted for publication 18 Dec. 2008.

Investigation partially funded by AGF98-0802 project from the Spanish Ministry of Education and Science. Seedling paternity analyses were carried out by J.M. Guerra-Sanz.

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number of pollen grains captured by the stigmas of the flowers, 20 fresh flowers per tree were collected 1 h after application from the tree directly receiving the pollen and from those trees behind it in Rows 2, 4, 8, and 16. The pollen grains adhered to the stigmas were counted under a microscope.

Seedling paternity. Seedling paternity was determined in 2000 in small samples of fruit randomly sampled from tagged shoots of the different pollination treatments. Mature fruits were harvested in November and the seeds germinated after cold stratification for a period of 4 weeks. When seedlings had formed the first two true leaves, they were collected and their DNA extracted. DNA from leaves of the two pollen donor cultivars (Hojiblanca and Arbequina) and the mother cultivar (Picual) was also extracted. Three microsatellite loci were used for paternity testing: DCA9 and DCA18 (Sefc et al., 2000) and AB0 (Guerra-Sanz, unpublished research). Microsatellite amplification was carried out by polymerase chain reaction with a Perkin-Elmer 9700 thermocycler (Perkin-Elmer, Waltham, MA) programmed following the protocol described in Guerra-Sanz (2002). All amplifications were resolved on 6% denaturing polyacrylamide gels and fragments were visualized using the Promega silver-staining kit (Promega, Madison, WI). Fragment length was determined by comparison with 10 bp DNA ladder markers (Invitrogen, Life Technologies, Carlsbad, CA) and/or DNA Molecular Weight Marker V (8 to 578 bp) (Roche Diagnostics, Mannheim, Germany). Electrophoresis results were analyzed with 1-D Manager analysis software (Tecnología para Diagnóstico e Investigación, Madrid, Spain).

Results

Cross-pollination deficit assessment. Pollination treatments significantly affected ($P < 0.001$) fruit setting in 'Picual'. In this regard, self-pollination reduced fruit set in every row in both years compared with open-pollination and mechanical cross-pollination (Fig. 1). On the contrary, no differences were found in open- versus cross-pollination in either year, with the only exception being the trees directly receiving the cross-pollen flow from the duster (Row 1) in Sorbas, in which a slight but significant increase in fruit set was noticed (Fig. 1). Cross-pollination also significantly increased fruit set with respect to open-pollination in Sorbas when analyses were performed taking each tree as a replication irrespective of the row (data not shown). Average fruit set under self-pollination was 0.1 and 0.2 fruits per panicle in Sorbas and Tabernas, respectively. Open- and cross-pollination more than doubled the levels of fruit set obtained by self-pollination. The ISI was 0.17 and 0.25 in Sorbas and Tabernas, respectively.

Pollen dispersal. Olive pollen density after a single mechanical application using a hand-duster on a calm day declined sharply with the distance from the application point (Fig. 2). Nonetheless, the number of pollen

Fig. 1. Fruit set at increasing distances (row) from hand-duster pollen application point in the different pollination treatments [self-pollination (SP), open-pollination (OP), cross-pollination (CP), and cross-pollination bagged (CPB)] Sorbas (A) and Tabernas (B) orchards. Mean separation by Tukey's test at $P < 0.05$.

grains deposited at 2 m was higher than the amount detected at 1 m (0.58 and 1.62 pollen grains/mm², respectively). The amount of pollen at 16 m was five times less than that captured at 2 m. Olive pollen was, however, still detected at 100 m at a similar amount to that observed at 16 m. The concentration of olive pollen grains significantly fitted an X-reciprocal model ($R^2 = 0.92$, $P = 0.0023$), omitting low pollen density recorded at 1 m, which is explained by the skip-distance effect, i.e., the maximum pollen deposition happens at a certain distance from an elevated pollen source (Jackson and Lyford, 1999). When a single mechanical application of pollen was performed against a flowering olive tree, cypress pollen was only recovered from the flowers of the tree directly receiving the application. In these flowers, a mean concentration of 50 pollen grains/mm² was recorded. Because the stigma of olive flowers has an average area of ≈ 3 mm² (Griggs et al.,

1975), an amount of ≈ 150 pollen grains per flower is inferred. No pollen grains were found in flowers from trees beyond 7 m.

Seedling paternity. The size of the alleles of the three microsatellite markers for putative pollen donors is presented in Table 1. The paternity of the seedlings was assigned to one of these cultivars considering their combination of alleles. Taking this into account, all seedlings coming from self-pollination were the result of self-fertilization, confirming the complete isolation of the flowers within the tissue paper bags (Table 2). In open-pollination, 70% of the seedlings were compatible with a self-fertilization process, 12% could be ascribed to 'Hojiblanca', and 12% to 'Arbequina'. An unknown cultivar seems to be the male parent of the remaining 6% of the seedlings. On the other hand, 70% of the seedlings obtained from cross-pollination were the result of cross-fertilization (36% from 'Hojiblanca' pollen and 34% from 'Arbequina' pollen). The

Fig. 2. Olive pollen dispersal after application with a hand-duster. Pollen was counted on plates placed at ground level.

Table 1. Alleles size (bp) of the three loci used for paternity analysis in the three known parental cultivars.

Cultivar	DCA18	DCA9	AB0
Picual	169/174	167/173	170/150
Hojiblanca	171/178	169/177	170/165
Arbequina	167/176	182/202	172

Table 2. Number of 'Picual' seedlings assigned to different pollen donors in the different pollination treatments [self-pollination (SP), open-pollination (OP), cross-pollination (CP)].

Treatment	Pollen donor			
	'Picual'	'Hojiblanca'	'Arbequina'	Unknown
SP	9	0	0	0
OP	12	2	2	1
CP	12	15	14	0

combination of alleles in the remaining 30% was compatible with self-fertilization.

Discussion

'Picual' behaves as a self-incompatible cultivar with a mean ISI of 0.21. This value means that 'Picual' forms on average five times more fruits under cross-pollination than under self-pollination. The preferential allogamy of 'Picual' has been demonstrated previously (Cuevas et al., 2001) and recently proven by paternity analyses of seeds (Díaz et al., 2006). 'Picual' self-incompatibility is in conflict with the existence of large monovarietal masses of this cultivar in Spain. According to official reports, 97% of the olive trees in Jaén (the world's largest concentration of olive) are of the cultivar Picual and to date pollination has never raised concern among Spanish olive growers. The solution to this contradiction comes from open-pollination results. Although both experimental orchards were monovarietal, open-pollination significantly increased fruit

set with respect to self-pollination up to levels equivalent to those of cross-pollination (Fig. 1). It is important to underline that the low fruit set in self-pollinated shoots was not the result of bagging, because the shoots that remained bagged all the time but received a single application of cross-pollen produced the highest number of fruits (Fig. 1). The absence of noticeable negative effects of tissue paper bags has been previously demonstrated (Del Río and Caballero, 1999).

Although the similar levels of fruit set suggest that open-pollination provided enough cross-pollen, the source of this pollen is a matter of discussion. The closest potential pollenizers to the experimental orchards were 250 to 500 m away depending on the location. The dispersal studies indicated that the concentration of pollen grains falls abruptly with the distance from the application point (Fig. 2). This is a physical law and pollen, like any other airborne particle (Whitehead, 1983), follows it. This is not to say that some olive pollen grains will not reach distant plants. Despite the rapid initial decrease,

pollen concentration falls much more slowly with increasing distance, which means that some pollen grains can reach greater distances. The same dispersal pattern and an effective pollination distance of 90 m were recorded by Griggs et al. (1975) in a young olive orchard. In fact, in our experiments, some olive pollen grains were captured 100 m from the source minutes after the hand-duster application. Long-distance dispersal of olive pollen grains up to 12 km has been observed under favorable weather conditions (González-Minero and Candau, 1997; Morettini and Pulselli, 1953). Pollination and fertilization with pollen from long distances has been proven in other wind-pollinated trees (Dow and Ashley, 1998; Polito et al., 2006; Squillace and Long, 1981). For this reason, isolation zones are recommended to avoid pollen contamination in wind-pollinated seed orchards and transgenic cultivars of crops such as maize (Sanvido et al., 2008; Squillace and Long, 1981). There are also reasons to believe that pollenizer trees might be more efficient than a single mechanical application of cross-pollen. First, a heavy flowering olive tree may form more than 50,000 million pollen grains (extrapolating data from Cuevas and Polito, 2004). Second, pollen from trees is dispersed gradually after wind gusts, which are probably more efficient than a single application using a hand-duster. Last but not least, given a final fruit set of just 1% to 2%, certain levels of cross-pollen can be enough for the usual yield in rainfed olive orchards. Wherever cross-pollen comes from, our results of seedling paternity analyses prove that cross-fertilization occurs in open-pollinated flowers (Table 2). This means that trees from neighboring plantations of different cultivars placed at least 250 m away are acting as unsuspected pollenizers. Cross-fertilization has been predominant in open-pollinated flowers in other studies on olive (Mookerjee et al., 2005), even under circumstances of higher isolation (Díaz et al., 2006). The high levels of self-fertilization found in open-pollinated flowers are, however, surprising. The small amount of fruit sampled for seedling paternity analyses may question this proportion, especially because the number of seedlings that emerged after cold stratification was low. A mentor or pioneer pollen effect is an alternative explanation for this apparent artifact (Visser, 1981), which requires further, more ample analyses.

Great benefits seem to be obtained from open-pollination in other Mediterranean countries with a long tradition of olive culture. In Italy, self-incompatibility of the main cultivars was demonstrated a long time ago, but pollination designs have rarely been adopted (Morettini and Pulselli, 1953). In Portugal, there are large isolated monovarietal orchards that bear normally without pollenizers (Almeida, 1940) despite the self-incompatibility of many Portuguese cultivars (Cuevas, 1992). The reconstitution of French olive cultivation after an episode of frost best illustrates the unsuspected benefits of open-pollination. In Feb. 1956, an intense frost killed many olive trees of different cultivars. New orchards

were planted based on a limited number of cultivars, some of them androsterile (Delmas et al., 1989). This decision turned out to be a mistake and revealed the self-incompatible condition of these cultivars. The reluctance of many olive researchers to accept the self-incompatible nature of olive is striking when the mere condition of being andromonoecious and anemophilous prove it: the formation of extra pollen in male flowers and its dispersion by wind can only be explained by the preferential allogamy of this crop.

The conclusion of this work is that the ample benefits from open-pollination will continue making pollination designs unnecessary in olive in Spain as long as its germ-plasm richness assures a constant flow of cross-pollen. However, the careless assumption of such a flow is unadvisable and the lack of pollination designs in areas of recent olive cultivation is a risk. In northern areas of California, isolated 'Manzanillo' orchards fail to produce acceptable yields, whereas in southern counties, the presence of 'Sevillano' trees seems sufficient to provide acceptable cross-pollination (Krueger, 2004). Despite the limited dispersal of pollen, the huge amount produced by olive trees suggests that pollination design by block assures sufficient levels of cross-pollination. In this regard, the association of small blocks of compatible 'Arbequina', 'Picual', and 'Hojiblanca', all high-yielding producers of reputed oils, seems convenient for extending the period of harvesting (Cuevas et al., 2001). On the contrary, the extension of super high-density solid orchards of 'Arbequina' to new areas of olive production may represent a risky venture.

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