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Production of total and stainable pollen grains in *Olea europaea* L

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Abstract

The production of the total and stainable number of pollen grains per flower and per inflorescence of 13 olive cultivars (*Olea europaea* L.) has been investigated. Grains stainability was evaluated by using the acetic carmine staining method whereas the production of pollen grains was calculated by using a Bürker haemocytometer. All pollen characteristics taken into account varied significantly among the cultivars. The number of flowers per inflorescence ranged from a maximum of 31.3 ('Cipressino') to a minimum of 13.0 ('Leccino'), the number of pollen grains per anther ranged from 123 346 ('Arbequina') to 40 975 ('Sant'Agostino'), and the pollen stainability from 97.6% ('Cipressino') to 48.0% ('Cellina di Nardò'). The number of stainable pollen grains per inflorescence averaged 2 559 512, ranging from a maximum of 3 913 678 ('Nociara') to a minimum of 940 143 ('Sant'Agostino'). All parameters were positively correlated, whereas a linear negative correlation was found between stainability and total pollen grains both per anther and per inflorescence.

Keywords: *Olea europaea*, pollen stainability, Bürker haemocytometer, pollen production

Olive trees produce small, white, either unisexual or hermaphrodite flowers, which are arranged in inflorescences of 15–35 flowers. The olive is an anemophilous plant and is generally known to produce pollen grains that are spread throughout the atmosphere by the wind buffeting the fruiting branches. Various studies have analyzed the pollen production, both in number and weight, in entomophilous (Oberle & Goertzen, 1952; Godini, 1981; Traynor, 1981; De Grandi-Hoffman et al., 1991; Szabó et al., 1996) and anemophilous species (Subba Reddi & Reddi, 1986; Tormo Molina et al., 1996; Hidalgo et al., 1999).

Since the 95% of worldwide olive acreage (i.e. more than 9 000 000 hectares) is located in the Mediterranean basin (Luchetti, 2002), large amounts of light olive pollen grains are dispersed in the late spring throughout the atmosphere of all the Mediterranean countries. Consequently, the pollen production has been evaluated for its possible relevance in human allergies (González Minero & Candau, 1997; Nieddu et al., 1997; Bicakci et al.,

2003; Syrigou et al., 2003), rather than for horticultural purposes. Thus far, the knowledge of the amount of pollen grains produced by a single anther of olive trees has received poor attention, and only few papers are available regarding this topic (Morettini & Pulselli, 1953; Villemur et al., 1984; Tormo Molina et al., 1996; Cuevas et al., 2001; Hidalgo et al., 2002; Orlandi et al., 2003). Moreover, the data reported ranged from about 3 000 pollen grains per anther (Morettini & Pulselli, 1953) to values about 30 times higher (Villemur et al., 1984; Tormo Molina et al., 1996; Cuevas et al., 2001; Hidalgo et al., 2002; Orlandi et al., 2003; Cuevas & Polito, 2004). On the other hand, it is very important from an agronomical point of view, but in general for pollen biology, to check pollen viability. Viability is the capacity to germinate and grow, but viable pollen grains may not germinate, *in vitro* or *in vivo*, if the conditions are not right (Dafni & Firmage, 2000). Several methods have been developed to test viability and other similar or more restrictive parameters such as stainability, pollen

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maturity, germinability, vigor, fertility (Dafni & Firmage, 2000). There are essentially four different methodologies to test viability: i) cytoplasmic stains; ii) enzyme reactions; iii) germination; iv) fruit and seed set (Nepi & Franchi, 2000; Dafni & Firmage, 2000). General cytoplasmic stains (lactophenol cotton blue, Alexander's stain, acetic carmine, neutral red, methylene blue, etc.) are easy and fast to use and are in some cases correlated with *in vitro* germination or fruit set (Dafni & Firmage, 2000). These cytoplasmic stains indicate that unstained pollen grains are dead because lack of cytoplasm but stained grains may be not fully fertile and an overestimation of pollen viability may occur (Nepi & Franchi, 2000). In particular, Alexander's stain can distinguish aborted from non-aborted pollen but tends to stain old and dead pollen and is not correlated with germination (Dafni & Firmage, 2000). Enzyme methods (tetrazolium, X-Gal, benzidine test, FCR, etc.), based on color reactions induced by enzymatic activities in the pollen grains, are based on the simple evidence that if an enzymatic reaction occurs the pollen grain is viable. The most important technique in this category is the fluorescein diacetate reaction (FCR) that also tests the plasmalemma integrity. This technique is simple and easy but needs to be performed very quickly and generally has the tendency to overestimate germination capacity and results may be very variable. The germination methods can be performed *in vitro* or *in vivo*. *In vitro* germination of pollen grains under appropriate conditions (temperature, culture medium) is rapid, simple and often correlated with fruit set and seed set but results can vary markedly depending on species and cultivars. *In vivo* germination simulates natural pollination and is generally verified by using a colorimetric detection of pollen tubes into the stigma (with aniline blue, lactophenol cotton blue, etc.), but results may be negatively influenced by factors such as incompatibility, competition, mass effects, minimum pollen load (Dafni & Firmage, 2000; Nepi & Franchi, 2000). The most effective method to test pollen viability is certainly fruit and seed set, but it is a time consuming and more qualitative than quantitative test (Dafni & Firmage, 2000). However, fertilization not only depends upon pollen viability, but also on the receptivity of the pistil, pollen deposition, etc. All methods present advantages and disadvantages and their use should be considered depending both on the species and the purpose of the study. Acetic carmine stain is a very fast a simple method to check the presence of the cytoplasm in pollen grains in order to ascertain the amount of normal pollen, i.e. stainable grains. This method can be used on large samples of pollen grains and allows normally

developed, mature pollen grains to be distinguished from the underdeveloped ones (Vassileva et al., 1991). It indicates the presence of structural elements that do not implicate the consequent viability of the stainable pollen grains but certainly do confirm the percentage of dead pollen grains (non-stainable) with respect to the total amount of pollen produced. Scarce references about olive cultivars (Mehri et al., 2003) were found concerning the stainability (Dafni & Firmage, 2000) of olive pollen grains intended as a marker of efficiency (normally developed pollen) in order to possibly determine the degree of pollen sterility of the cultivar (Stone et al., 1995). Actually, no information is available in the literature about number and stainability of pollen grains of many Italian olive cultivars.

From the biological point of view, most olive cultivars are self-incompatible and require cross-pollination for fruit setting. The choice of pollenizers among those genotypes producing large amounts of efficient pollen could be of strategic importance for fruit production and some cultivars might be preferred to others as pollen producers because of both compatibility and the more abundant masses of efficient grains produced and released in the air. This choice could be particularly important in new olive producing countries such as Australia and not be manifest in traditional areas in the Mediterranean basin.

The aim of this study was to evaluate the amount of total and stainable pollen grains per anther and per inflorescence produced by 13 olive cultivars to obtain useful data both for horticultural and allergological purposes.

Material and methods

Plant material

The study on 12-year-old olive trees was carried out in 2004 in one experimental olive grove in Palagiano, Apulia (Southern Italy). The trees were treated with the same cultivation practices, including irrigation, as those of commercial olive groves.

Eleven Italian and two Spanish olive cultivars were included in the present study, four of which are largely spread over more than 50 000 hectares in Italy, such as 'Cellina di Nardò', 'Coratina', 'Frantoio' and 'Leccino'. The varietal sample also included other slightly less important olive oil cultivars, such as 'Cima di Melfi', 'Cipressino' (syn. 'Frangivento') and 'Nociara'. The pickling cultivars 'Sant'Agostino' and 'Termite di Bitetto' and the two Spanish cultivars 'Arbequina' and 'Arbosana' were also tested. In addition, two intriguing Apulian cultivars, 'Dolce di Cassano'

and 'Pasola di Andria', pertaining to the so-called "sweet olive" group, were also included since they synthesize very small amounts of oleuropein, the bitter component of olives, and thus can be eaten directly after sizzling and salting.

Pollen collection

For each cultivar, five trees were randomly chosen at the blossoming stage, 20 inflorescences per tree were selected at random throughout the canopy in the middle position of fruiting shoots and the number of flowers counted. It is well-known that each small olive flower bears two anthers whose time of dehiscence is unpredictable; therefore, instead of single anthers, six samples of five flowers each per cultivar (i.e. 60 anthers) were removed from the inflorescences and placed in six vials (10 anthers $\times$ vial) closed with a gauze. The flowering and dehiscence of anthers were obtained at room temperature in the vials within 2–3 days. Successively, 4 ml of a 5% aqueous solution of polyoxyethylene-sorbitan monolaurate (Tween® 20) were added to each vial and the solutions were treated with an ultrasonic probe vibrating at 20 kHz for 25 s to facilitate the removal of pollen grains from the anthers.

Pollen counting

A Bürker haemocytometer was used for the counting of the pollen grains, according to a previously described methodology for almond (Godini, 1981) and slightly modified. Two drops of the sonicated suspension (4 ml) containing the pollen grains were placed with a micropipette onto each of the two main chambers of the Bürker haemocytometer. After placing the cover glass, pollen grains were counted in each of the nine small chambers forming the two main chambers. The number of pollen grains per anther was consequently calculated as the product of observed pollen grains in each small chamber by the dilution factor (0.1 μ l, dilution 1:40.000), divided by the number of anthers in each vial (i.e. 10).

Pollen stainability

To verify the incidence of the effective number of stainable grains, the acetic carmine staining method was used on six additional samples of 100 grains each per cultivar. After the dehiscence of the anthers, pollen grains were spread on a microscopic slide placed in a humidity chamber (a Petri dish), successively a drop of acetic carmine was placed on a new microslide and finally a small amount of hydrated pollen from each cultivar was suspended

and distributed uniformly in the drop. After placing a cover glass onto the microslide, the pollen grains were ready for the successive observation at the microscope. Pollen grains in selected microscopic fields were observed moving the preparation under the microscope in consecutive rows and from each field the total number of pollen grains was recorded to the final value of 600 pollen grains per cultivar on which the percentage of stainability was calculated.

Statistical analysis

Data were statistically analyzed by a one-way analysis of variance (ANOVA) at a $p \leq 0.01$ confidence level. The mean values were separated using the Student-Newman-Keuls (SNK) test. Correlations among the parameters examined were statistically evaluated by calculating the correlation coefficients (r) at different p -values.

Results and discussion

The average number of flowers per inflorescence was 19.6 which is similar to the value reported in the literature for a number of olive cultivars. The number of flowers varied greatly among cultivars, from a minimum of 13.0 ('Leccino') to a maximum of 31.3 ('Cipressino'), and a half of our varietal samples exhibited more than 20 flowers per inflorescence (Table I).

The average number of pollen grains per anther was 79 802 ranging from 5 to 15% lower than the numbers reported for distinct varietal populations (Villemur et al., 1984; Tormo Molina et al., 1996; Hidalgo et al., 2002; Orlandi et al., 2003; Cuevas & Polito, 2004) and over 25 times higher than those

Table I. The mean number of flowers per inflorescence and percentage of pollen stainability.

Cultivar	Flowers/Inflorescence (No.)	Stainability (%)
Arbequina	13.4 E	90.6 CD
Arbosana	15.5 DE	76.5 E
Cellina di Nardò	25.6 B	48.0 F
Cima di Melfi	22.1 C	95.2 AC
Cipressino	31.3 A	97.6 A
Coratina	17.4 D	91.8 CD
Dolce di Cassano	17.7 D	53.0 F
Frantoio	23.2 C	96.6 AB
Leccino	13.0 E	93.8 BC
Nociara	23.7 BC	90.2 CD
Pasola di Andria	21.8 C	94.6 BC
Sant'Agostino	13.2 E	86.8 D
Termite di Bitetto	17.0 D	90.4 CD
Mean value	19.6	85.0

Capital letters refer to $p \leq 0.01$, as obtained by the SNK test

indicated by Morettini and Pulselli (1953). Unfortunately, Villemur et al. (1984) only referred that the measurements were conducted with the support of the Laboratory of Palynology at Montpellier without additional specifications, whereas Morettini and Pulselli (1953) did not elucidate the procedures by which their data were obtained. Both Tormo Molina et al. (1996) and Hidalgo et al. (2002) used the method proposed by Cruden (1977), whereas Orlandi et al. (2003) and Cuevas and Polito (2004) used different methods fully described in their papers. Nevertheless, in Italy, the number of 3 000–10 000 grains per anther has been assumed as the most reliable for decades, although the estimation method was not specified (Baldini, 1986). Three cultivars, 'Arbequina', 'Arbosana' and 'Cellina di Nardò', showed a production of pollen grains per anther higher than 100 000 and 'Sant'Agostino' alone exhibited a production lower than 50 000 grains per anther (Figure 1). When considering the total number of pollen grains per inflorescence (Figure 2), 'Cellina di Nardò' was the most productive with 6 295 357 grains, followed by 'Nociara' with 4 338 889; 'Sant'Agostino' produced only 1 083 114 grains due to the scarce number of both flowers per inflorescence (13.2) and grains per anther (40 975). The average value was about 3 million grains which is similar to the average values reported by Villemur et al. (1984), Tormo Molina et al. (1996) and Hidalgo et al. (2002), with 2, 3 and 4 million grains, respectively.

The pollen stainability (Table I) was over 90% in nine cultivars, with 'Cipressino' showing the highest absolute value (97.6%). 'Dolce di Cassano' was characterized by low stainability values (53.0%) and the absolute lowest pollen stainability was 48.0% recorded by 'Cellina di Nardò'. Therefore, it seems possible to affirm that 'Cellina di Nardò' is genetically prone to produce large amounts of grains per flower without having the possibility to adequately feed all of them with the result that more than one half of the grains are not normally developed and thus 'stainable'. However, in olive a (cytoplasmic)-male sterility with a consequent variety of pollen abortion stages may occur, especially at the microspore stage after tetrad formation (Villemur et al., 1984; Besnard et al., 2000), but a genetic male sterility cannot be totally excluded (Besnard et al., 2000). Various male sterile cultivars have been identified by morphological and genetic analyses, such as 'Cerasola' (Baldini & Guccione, 1952), 'Olivière', 'Lucques', 'Tanche' (Villemur et al., 1984; Besnard et al., 2000); male sterility has been also detected in oleaster trees in Spain, Tunisia and Sardinia (Amane et al., 1999). The low

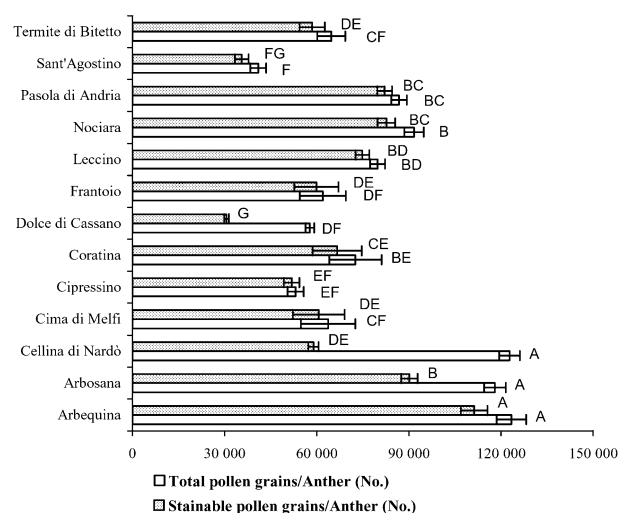


Figure 1. The mean values of total pollen grains per anther and stainable pollen grains per anther in different olive cultivars. Capital letters refer to a difference significant at $p \leq 0.01$, according to the SNK test. The horizontal line on each bar indicates the standard error for the six replicates.

stainability values of 'Cellina di Nardò' and 'Dolce di Cassano' may indicate a possible partial male fertility behavior of these two cultivars, but further investigations on pollen formation are needed to better elucidate their biology. When we consider the single anther as a reference unit (Figure 1), only 'Arbequina' and 'Arbosana' produced more than 90 000 stainable grains per flower (111 258 and 90 180, respectively); if the number of flowers per inflorescence is used as a reference unit (Figure 2), only 'Cellina di Nardò', 'Cipressino', 'Nociara' and 'Pasola di Andria' produced more than 3 000 000

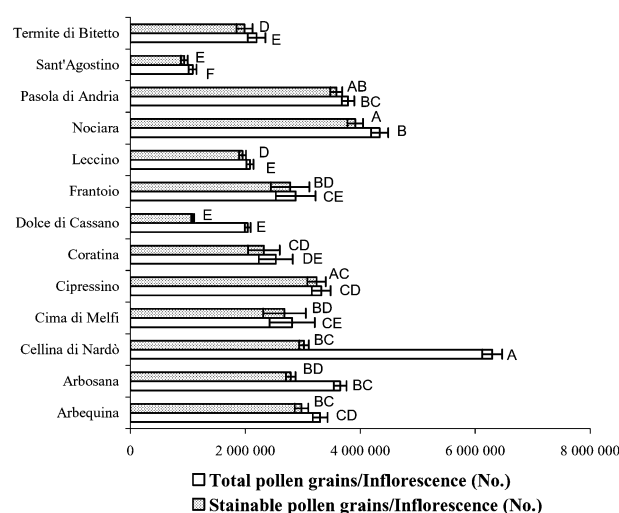


Figure 2. The mean values of total pollen grains per inflorescence and stainable pollen grains per inflorescence in different olive cultivars. Capital letters refer to a difference significant at $p \leq 0.01$, according to the SNK test. The horizontal line on each bar indicates the standard error for the six replicates.

stainable grains per inflorescence (3 021 771, 3 241 882, 3 913 678 and 3 583 467, respectively). 'Dolce di Cassano' and 'Sant'Agostino' produced the lowest number of stainable pollen grains both per anther (30 609 and 35 567, respectively) and per inflorescence (1 082 543 and 940 143, respectively).

Without taking into account the pollen stainability, the amount of pollen grains per anther and/or per inflorescence of our varietal sample generally agreed with the values reported in the literature already mentioned; when the parameter "stainability" is included, almost none of our olive cultivars seemed capable of producing such a high amounts of stainable (i.e. normally developed) pollen grains per anther and/or per inflorescence. On the whole, our varietal sample was able to produce about 66 000 and about 2 500 000 of stainable pollen grains per anther and per inflorescence, respectively. Differences among cultivars in flower quality and pollen stainability may be attributed to both physiological and environmental effects, but a genetic influence has to be markedly considered. The acetic carmine method used in the present work allowed us to analyze a large sample of pollen grains belonging to 13 cultivars in a fast and simple way in order to obtain data about stainable (normally developed) pollen grains for each cultivar.

The results of the present study showed a high variability among cultivars for pollen production. Accordingly, the high pollen production of this species could be interpreted as the necessity of helping the casualness of the cross-pollination when the wind is sole pollen vector. The general capacity to produce pollen is genetically and physiologically controlled; the differences among cultivars observed in the present study are certainly indicative of the intervarietal variability, as detected in other fruit species, i.e. apple, peach, plum, grape, almond, sour and sweet cherry (Oberle & Goertzen, 1952; Godini, 1981; Szabó et al., 1996).

Olive has both perfect and staminate flowers, the amount of which is characteristic for each cultivar and can vary from year to year depending on developmental and environmental conditions. Pollen produced by staminate and hermaphrodite

olive flowers does not differ in amount, size and quality, and an increase in pollen production at the plant level is proposed as the likely role of staminate flower in olive (Cuevas & Polito, 2004). The level of fruit set is determined by the tree-environment relations and is mostly independent of the amount of staminate flowers formed (Lavee et al., 1996). Furthermore, pistil abortion may have a little influence on the male component of flowers because it generally occurs around a one month before bloom during the phase of rapid pistil growth, by which time stamen development is well advanced (Cuevas et al., 1999). Concerning the pollination efficiency of olive cultivars in general, the pollen stainability could be an important characteristic to be taken into consideration as an indicative parameter of pollen development and/or structure and/or degree of (male)-sterility. Olive cultivars producing the greatest amount of stainable and compatible pollen grains per inflorescence could be preferred when choosing potential pollen donors particularly in new olive producing countries, and this study highlighted some remarkable differences among cultivars in terms of stainable pollen. Nevertheless, climatic conditions strongly influence all the pollination and fertilization process. In particular, the growth of the pollen tube can be limited by high and low temperatures during flowering and consequently if pollen tube is slow the sac can degenerate before the tube reached it (Griggs et al., 1975). Furthermore, when considering the alternate bearing in olive, no clear morphological differences between flowers in the 'on' and 'off' years have been observed, except sometimes in flower size (Lavee et al., 1999). A probable explanation of the low percentage of olive flowers setting fruit on abundantly flowering trees ('on' year) is the control exerted by the first-to-set developing fruits, which prevent other fruits developing in their vicinity (Cuevas et al., 1995; Lavee et al., 1999). However, inflorescence and flower number on a shoot basis could not be considered a major limiting factor of fruit load (Lavee et al., 1999).

The evaluation of the relationships among the parameters tested permitted us to emphasize that the *r* values (Table II) were highly significant in almost

Table II. Correlations among the different parameters examined in the study.

	Grains/Anther	Stainable grains/ Anther	Grains/ Inflorescence	Stainable grains/ Inflorescence	Flowers/Inflorescence
Stainability	-0.338-0.002	0.224-0.049	-0.338-0.002	0.250-0.027	0.093-0.420
Grains/Anther		0.806-0.000	0.760-0.000	0.598-0.000	-0.089-0.436
Stainable grains/Anther			0.460-0.000	0.703-0.000	-0.183-0.108
Grains/Inflorescence				0.777-0.000	0.517-0.000
Stainable grains/Inflorescence					0.524-0.000

Each pair of data indicates the *r* value and the probability ($p > |r|$), respectively

all cases, as expected. A linear negative correlation ($p \leq 0.01$) was found between stainability of pollen grains and total pollen grains both per anther and per inflorescence, suggesting that olive trees are unable to sustain an unlimited amount of normally developed pollen grains, the most evident example being 'Cellina di Nardò'.

It is well-known that the optimal fruit set in olive trees is about 2.5% (Morettini, 1939; Cuevas et al., 1995; Lavee et al., 1996) which means that trees producing 50 kg of drupes weighing 2 g each are able to differentiate up to one million flowers; it also signifies that, for such olive trees, the production of a hundred billion pollen grains is the rule.

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