

Antigenic and allergenic differences between 16 different cultivars of *Olea europaea*

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In southern Spain, during the months of May and June, most of the air-borne pollen is from olive trees. However, in inland regions, such as Madrid, there exist many atopic patients who exhibit positive reactions to pollen of gramineae and reactivity to *Olea europaea* pollen. It is remarkable that only a few of them are exclusively reactive to *Olea europaea* (monosensitized), in contrast to southern zones, in which we found an increase in monosensitization (1).

In 1984, González Quevedo in our department began to study *Olea europaea* pollen from different cultivars. Using both *in vitro* and *in vivo* techniques, we found statistically significant differences in allergenic activity and potency between the different varieties. Direct RAST and skin tests revealed greater allergenic activity in extracts of the varieties cornezuelo, cornicabra, gordal, ojiblanco, manzanilla, and verdial when compared with the other varieties studied. In addition, we confirmed that the major allergen of *Olea europaea* pollen has an isoelectric point with value close to 6. This major antigen is present in all the analysed extracts from the distinct 16 cultivars, and we found that the allergenic reactivity increased in parallel with the increase in content of this antigen (2).

Cross-reactivity, caused by the presence of allergenic fractions common to extracts of different origin, has been widely studied using inhibition RAST and, less frequently, by CRIE (radioimmunoelectrophoresis) and reverse

enzymimmunoassay. Bousquet et al. studied the cross-reactivity between four types of Oleaceae (*Olea europaea*, *Fraxinus excelsior*, *Ligustrum vulgare*, and *Phillyria angustifolia*) using inhibition RAST, isoelectrofocusing, and tandem crossed immunoelectrophoresis (CIE). They detected a high degree of cross-reactivity but not a total identity between these four pollen species (3).

In 1992, Baldo et al. studied the cross-reactivity between olive and privet pollens, with olive pollen having major antigens between 18 and 19 kDa, 20 kDa, and 40 kDa, while pollens in privet were in the range of 19, 20, and 40 kDa. Baldo et al. concluded that the presence of IgE antibodies that react with the pollen may not necessarily be a consequence of the sensitization to the pollen of these species (4).

In 1984, our group studied 16 cultivars of *Olea europaea* from the botanical and agronomic point of view (5–14). This comprised a biological evaluation *in vivo* and *in vitro*, and a study of the protein content of the pollen extracts, aimed at establishing whether there are distinctive characteristics among the different cultivars.

Materials and methods

The trees studied belong to the School of Agriculture, Seville. From the total of 39 cultivars, 16 were chosen for their pollen production in earlier years.

The botanical and agronomic study was based on the following essential characteristic:

- characteristics of the tree – height, vigour, shape (of canopy);
- characteristics of the main branches – length, vigour, direction;
- characteristics of the secondary branches – length, interknot distance, direction;
- characteristics of the inflorescence – density, orientation;
- characteristics of the drupe – shape, size, mean weight, change of colour during ripening;
- characteristics of the endocarp (pit) – shape, surface aspect (colour, roughness), number of striations.

Pollens for the microscopy study were collected (15) in bags of natural Kraft paper of 500 × 300 mm plus 80 of gusset, placed for 3 days on various branches of the trees being studied.

The untreated polliniferous material was sieved free of impurities. It was then studied by direct-light optical microscopy at ×100, and by scanning electron microscopy at various magnifications (from ×1000 to ×20 000), both in line with the method described at the first APLE congress (16).

For biological evaluation of the pollens of the different cultivars, untreated pollen was collected, then finely sieved and tested for sterility. The major protein constituents were extracted from the non-defatted pollen material following the modified technique of King: maceration in Frugoni's liquor, filtration, dialysis in distilled water, and adjustment of protein concentration in line with the method of Lowry. Two types of diagnostic extract were obtained: one with glycerine-phenol, for *in vivo* tests, and the other lyophilized for *in vitro* tests.

The study was conducted in 20 patients selected from the olive-growing areas of Seville and Córdoba. All presented rhinitis and asthma, with positive-prick to a batch of commercially produced mixture of *Olea* denoted as 'Olea O'. In addition, all presented IgE serum levels above the normal for their age. Despite most of our patients had a positive RAST (class 3 or 4) when tested with commercial discs sensitized with an extract denoted as 'Olea X'. With 15 selected patients who collaborated until the end of the study, we prepared a pool of sera for inhibition RAST. None of the patients had previously received immunotherapy with olives.

During the study, they were subjected to the following protocol:

- close clinical monitoring and routine analysis;
- determination of total serum IgE, using an enzymatic technique;

- skin test with a commercial mixture of *Olea* (Olea I and Olea II), using the prick technique;
- determination of specific IgE against a commercial *Olea* mixture (Olea X, Olea II, Olea III and Olea IV), using the RAST technique;
- skin test with cultivars, using the prick technique;
- determination of specific IgE against cultivars by the RAST technique, using discs activated with BrCN following the method of Ceska and later sensitized with our extracts.

An evaluation was made of the allergenic activity and potency of each extract (prepared from the pollen of each of the olive groves in the study), of a commercial extract of *Olea europaea*, and one of variety Picual grown in its region of origin (Jaén), using inhibition RAST with the pool of sera from the 15 selected patients.

A physico-chemical characterization was carried out on the 16 pollen extracts of the cultivars under study, on a commercial extract of *Olea europaea*, and on the Jaén Picual extract, by isoelectrofocusing on a horizontal plate of polyacrylamide gel. The results were compared with the data obtained from pricks and direct RAST, in order to draw better conclusions. We classified the cultivars as 'GOOD', 'MEDIUM', and 'POOR', according to the following criteria.

- *Prick*: We denoted as 'GOOD' and 'POOR', respectively, those cultivars situated above and below the range constructed for the mean of the positive responses (mean = 51.58), with a standard deviation (SD) of 19.33. MEDIUM varieties are those within that range, although some of them were close to the limiting groups.
- *Direct RAST*: In the same way, we constructed a range for the mean total radioactivity (mean = 37.27) with SD = 13.11.
- The criteria of classification for the inhibition RAST are provided by the number of microlitres of extract necessary to inhibit 50% of the RAST: POOR, more than 100 µl, MEDIUM–POOR, 50 µl, GOOD, 15–30 µl; and VERY GOOD, 5–10 µl.
- Protein analysis by isoelectrofocusing enabled us to classify the 16 cultivars by the pattern of the protein spectrum and size and situation of the bands.

Results

Agronomic study

- Family, Oleaceae; genus, *Olea*; species, *Olea europaea* L.; common name, olive; subspecies,

O.E. *Oleaster*; Wild olives, O.E. *Sativa*. Cultivated olives and certain spontaneous olives with characteristics similar to those of cultivated olives.

- *Cultivars*: The cultivated olives present special features typical of the ecotype, i.e. typical of the environment (climatological and soil conditions) in which each tree grows. Thus, we speak of cultivar rather than variety.

- 1 Budiego;
- 2 Cañivano blanco (O.E. *Alconii* M.R., O.E. *Alba risso*);
- 3 Cañivano negro (O.E. *Garciae* M.R.);
- 4 Carrasqueña (O.E. *Columella* M.R.);
- 5 Cornezuelo of Jaén (O. E. *Ceraticarpa clem.*);
- 6 Cornicabra (O.E. *Cavanillesii*);
- 7 Gordal (O.E. *Reaglis clem.*);
- 8 Hojiblanco (O.E. *Arolensis clem.*);
- 9 Lechin (O.E. *Ovalis clem.*);
- 10 Manzanilla (O.E. *Pomiformis clem.*);
- 11 Negral (O.E. *Tenax clem.*);
- 12 Picual (O.E. *Rostrata risso*);
- 13 Rapazaya or Rapa-sayos (O.E. *Herreros* M.R.);
- 14 Verdial of Pilas and Verdial of Vélez (O.E. *Viridula gouan*);
- 15 Zorzaleña (O.E. *Argentata clem.*).

Characteristics of the plant: Evergreen tree 6–10 m in height, with thick, knotty, and twisted trunk covered with brownish-grey, heavily fissured bark. The canopy is dense and rounded, with abundant flexible branches. The leaves are simple and opposed, coriaceous, elliptical, oblong or lanceolate, 3–9 cm in length, short petiolate, with strongly green upperside and paler underside. Stalks and shoots are also pale. The flowers, grouped on axial racemes, are small, pale, and odorous with two stamens. The fruit is the olive, a fleshy ovoid drupe, 1–3.5 cm in length, with very oily pericarp and hard endocarp, green or black in colour, with a single seed.

The botanical and agronomic characteristics of the different cultivars enable their differentiation, as there are significant differences between them.

Optical and electron microscopy study

We studied anemophilous pollen, with an average diameter of 25 µm; the shape was oval prolate and tricholporate; thick exine, reticulated and thin intine with uncus.

Optical microscopy showed no major differences between the pollens of the different varieties, although it could be seen that the thickness of the exine varied between varieties.

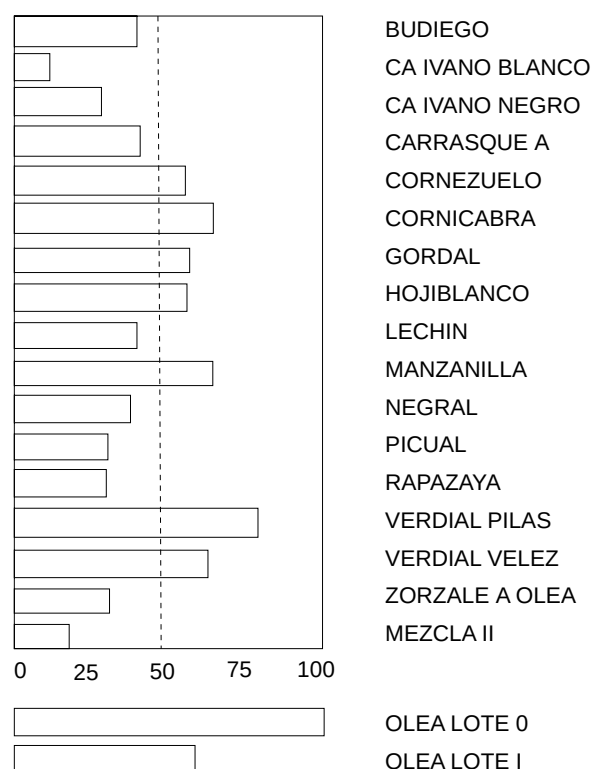


Figure 1. Percentage of positive prick tests for extracts obtained from different cultivars.

Scanning electron microscopy at different magnifications showed differences in the distances between the spicula of the exine and in the size of the lumens present in the exine of the different olive varieties.

Biological evaluation

Figure 1 shows a histogram (units of per cent) from a positive prick test obtained in 20 patients with extracts from each cultivar. Considerable reactivity of response can be seen for the cultivars Cornicabra, Manzanilla, Verdial of Pilas, Verdial of Vélez. We also found an enormous variability between the three batches of commercial Olea mixtures, ranging from 100% for Olea 0 to more than 18% for Olea II, with Olea I being intermediate.

For the direct RAST with cultivars, Fig. 2 illustrates the response of the final group of 10 patients, expressed as percentage of total radioactivity. The strong positives of Carrasqueña, Cornezuelo, Cornicabra, Negral and Picual are noteworthy, although none of the cultivars showed responses markedly outside the mean range. It can also be seen that the peak PiI, i.e. for

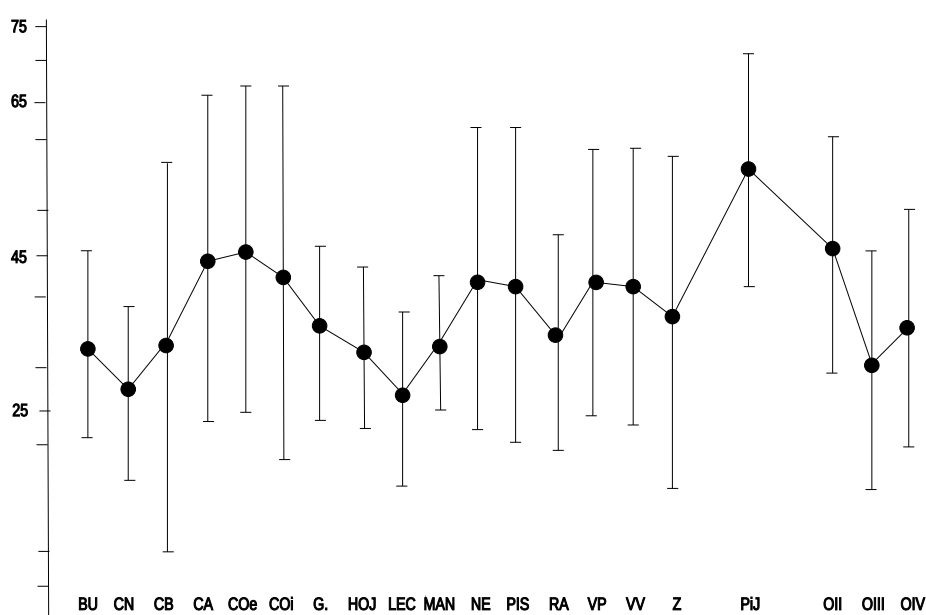


Figure 2. Average values (cpm) and standard deviation reached by each of the varieties of pollen of olive tree.

Table 1. Response in direct RAST to different extracts of olive tree pollen

	Olea X	Olea II	Olea III	Olea IV
RAST 4	50%	90%	50%	70%
RAST 3	30%		20%	10%
RAST 2,1,0	20%	10%	30%	20%

an extract of Picual grown in Jaén, clearly exceeds that of PiS, i.e. our Picual grown in Seville.

When we tested the four batches of commercial mixtures of Olea (Olea X, Olea II, Olea III, and Olea IV) in the same patients using direct RAST, although there was no 100% correlation from one batch to another, we did find that there was not a great disagreement between them in the prick tests, because the positives in the RAST were between 70 and 90% for the four batches (Table 1).

Table 2 shows the prick-RAST correlation compared with the results of the inhibition RAST and isoelectrofocusing, following the criteria set out in the section 'Material and methods'.

Discussion

We use the idea of cultivars rather than local varieties or subvarieties, because a variety is a group of individuals belonging to the same species and

Table 2. Reactivity to several extracts using different methods of evaluation

	Prick test	Direct RAST	Inh. RAST	Isoelectrofocusing
Good	Cornicabra Verdial P.		Olea II Verdial P. Verdial V. Cornicabra Cornezuelo	Cornicabra Verdial P. Verdial V. Rapazaya Manzanilla Cornezuelo Carraqueña Picual
Average	Verdial V Gordal Manzanilla Olea I Cornezuelo Hojiblanco Budiego Carrasqueña Lechin C. Negro Zorzaleña Rapazaya Picual Negral	Cornezuelo Olea II Carrasqueña Negral Cornicabra Picual Verdial P. Verdil V. Zorzaleña Gordal Olea IV Budioego Rapazaya Manzanilla C. Negro Olea III C. Blanco Lechin	Picual Rapasqueña Rapazaya Manzanilla Gordal Hojiblanco Negral Zorzaleña Lechin	Negral Hojiblanco Zorzaleña Olea II
Bad			Budiego C. Blanco C. Negro Lechin	Budiego C. Blanco C. Negro Lechin Gordal
	Cañ. Blanco Olea II			

having similar characteristics, and the cultivated olives present special features that depend on their adaptation to the environment or ecotype. This, and the confused common naming used, giving different names to a single variety or the same name to distinct varieties, lead to great confusion in the identification of each variety. Several authors (17,18) emphasize the importance of carrying out the studies in experimental fields where growth takes place under the same climatological and soil conditions. Our study has been carried out in the Seville School of Agriculture, where tree development is almost totally natural, because there is currently no crop management. Nevertheless, we have worked on trees that correspond to the cultivars described, based on the data in the literature consulted (5–14) and on the advice of the school's experts.

Having defined these terms, we have to say that, on analysing the chosen cultivars, we found agronomic differences from those described in the literature (18).

In the microscopic study of the pollens, the question is not so clear. Optical microscopy revealed some different features relating to the thickness of the exine. However, for the moment, we cannot draw any conclusion. This is also the case for evaluating the differences observed with the scanning electron microscope. Nevertheless, at first sight – and particularly in certain cultivars (hojiblanco) – there seem to be differences in crest size and shape, and in lumen size in the reticule of the different pollens.

Regarding the biological evaluation, in the analysis of our results, we think it of interest to emphasize the great breadth of prick response for the varieties Cornicabra, Manzanilla and the two Verdials studied, and the great variability shown by the different batches of commercial mixtures of *Olea* tested (Fig. 1), which are not greatly different from the RAST results (Table 1). In the direct RAST, the strong positives that stand out (Fig. 2) are those of Carrasqueña, Cornezuelo, Cornicabra, Negral, and Picual, although none lies far outside the mean range. It is curious that the peak PiJ, i.e. for the extract of Picual grown in its native area (Jaén), far exceeds our Picual (PiS), which is grown in Seville. This raises the question of species ecotype when evaluating its activity.

Conclusions

- 1 There are obvious differences between the different cultivars from the agronomic point of view, following the direct examination of the plant – even more so when the fruits are compared.
- 2 We have been able to corroborate that the environment and crop management are factors able to induce qualitative changes, allergologically fundamental in a particular variety or cultivar.
- 3 The optical microscopy study shows apparent differences between the various pollens. However, these would have to be confirmed by statistical micrometric methods.
- 4 Similarly, micrometric methods would have to be used to confirm the differences between the pollens revealed by electron microscopy.
- 5 Cornicabra, Verdial of Pilas and Verdial of Veléz are cultivars with acceptable levels of quality. This group also includes Manzanilla, Cornezuelo, and Carrasqueña. We classify Cañivano blanco, Cañivano negro, Lechín ecijano and Budiego as poor cultivars.

In 1994, following the line of research begun some years earlier in our Allergy Unit, we isolated, characterized, and standardized the pollen extracts of 12 varieties of *Olea europaea*: Alameño, Aloreña, Azuejo, Blanqueta, Budiego, Callosina, Manzanilla, Negral, Picual, Rapasaya, Zarzario, and Zorzaleña.

The method of collection used is appropriate for their study and subsequent analysis, because they are obtained with 98–99% purity.

Inhibition RAST shows no significant differences in allergenic potency between the varieties Zorzaleña, Rapasaya and Alameño.

Immunoblotting shows significant differences in allergenic behaviour in the 12 varieties of *Olea europaea* pollen studied. Three groups could be separated: those of greatest allergenicity, represented by the varieties Picual, Negral, Zarzario, Alameño, Azuejo and Rapasaya; those of moderate allergenicity, represented by Budiego, Blanqueta and Manzanilla; and those of scarce allergenicity, i.e. Aloreña, Callosina, and Zorzaleña.

Some 75% of the varieties of olive pollen studied presented molecular weights of approximately 17 000, 19 000, and 42 000 Da, protein bands with well-fixed IgE, by immunoblotting. These would correspond to the location of the main antigens of olive pollen.

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