

Original article

Allergenicity and immunochemical characterization of six varieties of *Olea europaea*

Background: The inhalation of *Olea europaea* pollen is one of the most important causes of allergic respiratory diseases in the Mediterranean basin. The objective of this study was to investigate the antigenic and allergenic composition of six different *O. europaea* varieties collected in southern Spain.

Methods: The varieties included in the study were: Acebuche (wild olive), Carrasqueño, Nevado, Hojiblanco, Manzanillo and Picual. Extracts of these six varieties were prepared. Twenty-nine olive individuals with an immunoglobulin(Ig)E-mediated allergy to olive pollen were skin tested with these extracts. The antigenic profile of these extracts was evaluated by SDS-PAGE; the allergenic profile was investigated by immunoblotting using the serum of these 29 individuals. The Ole e 1 content was established by ELISA inhibition using purified Ole e 1 and rabbit polyclonal antibodies and by scanning densitometry.

Results: The extracts that induced the smallest wheal size were Acebuche and Hojiblanco, being significantly different from the rest of the extracts. The antigenic and allergenic profiles of the extracts also varied. The Ole e 1 content ranged from 0.050 in Hojiblanco to 0.232 in Manzanillo, measured by ELISA inhibition and from 0.153 in Hojiblanco to 0.677 in Nevado, measured by scanning densitometry.

Conclusions: The different varieties of *O. europaea* pollen studied demonstrated great differences in the *in vivo* and *in vitro* potency of the extracts. There were significant differences in the Ole e 1 content, while the protein content remained very similar in these extracts. This study confirms previous observations of a great variability in the antigenic and allergenic composition of *O. europaea* pollen extracts and establishes significant differences in Ole e 1 content.

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Olea europaea (olive tree) is one of the most common and economically important trees in the Mediterranean area. It is also widely cultivated in other areas of the world, such as some countries in South and North America. During the spring period, olive trees produce large quantities of pollen and, in some areas such as Andalucía in Spain, olive pollen is the most abundant pollen in the air, followed by grass pollen. Recently, the olive tree has also been introduced as an ornamental tree in many countries.

It is well established that the inhalation of olive pollen induces sensitization and symptoms in genetically susceptible individuals. Nine allergens have been described so far and several more await discovery. In 1998 Boluda et al. (1), described the allergenic composition of different varieties of olive pollen collected in Spain and in the US, and established qualitative and quantitative differences in the concentration of some allergens. It seems that pollen recollected in Spain contained more Ole e 1 and less Ole e 4. Studies

in Israel (2) also suggest the different allergenic composition of olive pollen collected from different cultivars. This variability in the allergen composition was also suggested by Barber et al. (3). Two doctoral theses have been devoted to the study of the allergenicity of different varieties of olive pollen in Spain (4, 5).

Approximately 260 varieties of olive trees are registered and distributed throughout Spain (6). These varieties have been selected based on their adaptation to ecological factors, such as climatic and soil conditions. The classification of these varieties was based, for a long time, on morphological and agronomical characteristics such as the shape of the fruit and the leaves (7). More recently, the analysis of isoenzymes has been used to confirm the previous classification. Currently, the DNA analysis provides a better tool to investigate and compare the genetic material of the different varieties of the olive tree (8).

The aims of this study were to investigate and characterize the antigenic and allergenic composition

of six widely distributed varieties of olive pollen recollected in Spain during 1998. The varieties analyzed were: Carrasqueño, Picual, Acebuche (wild olive), Nevado, Hojiblanco and Manzanillo. The Ole e 1 content was measured in each extract and the allergenic potency was established by skin testing and *in vitro* assays.

Material and methods

Allergen extracts

Six different varieties of *O. europaea* pollen were collected in Andalucía, Spain in 1998 by expert personnel trained in the identification of olive tree varieties and in the collection of pollen. The origin of the different pollen collections was stated in the raw material certification document. All pollen samples had a purity greater than 90% (Mediterráneo Polen, Jaén, Spain). The six varieties were Carrasqueño, Acebuche, Manzanillo, Hojiblanco, Nevado and Picual. All six extracts were prepared following the same standardized protocol. Briefly, 6 g of pollen were diluted in 1 : 5 wt/vol in phosphate-buffered saline (PBS) 0.01 M (pH 7.4) and extracted 4 h at 4°C under continuous magnetic stirring. After homogenization, the extract was centrifuged at 16,266 g at 4°C for 30 min and the supernatant was collected. Precipitated was again reconstituted in 1 : 5 wt/vol in PBS 0.01 M (pH 7.4) and extracted overnight at 4°C with continuous magnetic stirring. Afterwards, the extract was centrifuged at 16,266 g. The supernatant was separated and mixed with the first extraction, sterile filtered and dialyzed against bidistilled water for 20 h in dialysis membranes of 3.5 kDa (Spectra/Por Membrane). The extract was then filtered, frozen and lyophilized.

Patient population

Twenty-nine individuals from the Madrid region with clinical symptoms after the inhalation of *O. europaea* pollen were included in the study. All patients suffered from IgE-mediated intermittent allergic rhinoconjunctivitis and/or allergic asthma. These patients met the criteria outlined by Johansson et al. in a position paper recently published (9). A prick test solution was prepared with each variety of *O. europaea* at a final concentration of 2 mg of lyophilized material/ml. All patients were skin-prick tested in duplicate in the forearm, and the wheal was measured after 15 min. A serum sample was collected and stored at -20°C. A serum pool was prepared with the individual sera.

Another serum pool was prepared with the serum of 15 *O. europaea* skin test positive individuals residing in Jaén (Andalucía).

Protein content

The protein content of six varieties was measured by the Lowry–Biuret method (Sigma Diagnostics, St Louis, USA), following the manufacturer's instructions.

Sds-page

SDS-PAGE analysis was used to determine the antigenic profile of the extracts. The samples were run in SDS-PAGE gels with 2.67% C, 15% T acrylamide as described by Laemmli (10). The samples were denatured at 100°C for 10 min and centrifuged for 1 min at 16,266 g. Fifty µg of lyophilized material was loaded on the gels. Reference markers with known molecular weights (Bio-Rad Laboratories, CA, USA) were run in the same gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories).

The antigenic profile was studied with the Scanner Sharp JX-330 and analyzed with Image Master 1-D Elite v 2.01 Pharmacia Biotech (Barcelona, Spain).

Immunoblot

The separated proteins were electrophoretically transferred to P-Immobilon membranes (PVDF) (Millipore, Bedford, USA). After transfer, the membranes were dried at room temperature for 4 h. Two types of serum pools were used; one containing sera from 29 patients from the Madrid area and the second pool containing sera from 15 patients residing in Jaén.

The membranes were incubated overnight with both pools of sera, individually, in PBS 0.01 M Tween 2%. Specific IgE binding was detected with peroxidase-conjugated monoclonal antihuman IgE (Ingenasa, Madrid, Spain) for 2 h. The allergenic profile was studied with the Scanner Sharp JX-330 and analyzed with Image Master 1-D Elite v 2.01 Pharmacia Biotech.

This experiment was conducted in order to establish if there were differences in IgE-binding patterns between the two serum pools.

Biological activity of the extracts

The potency of the six extracts was evaluated by a modified ELISA competition assay, previously described by Casanovas et al. (11). A standardized *O. europaea* extract (C.B.F. LETI S.A. Madrid, Spain) was used as control to evaluate the potency of each extract. The results were expressed in HEP (biological unit) per g of raw material.

ELISA inhibition

The control *O. europaea* extract and the six varieties were tested by ELISA inhibition to evaluate their allergenic potency *in vitro*. Plastic microtiter plates (Immulon IV, Dynex Technologies, Chantilly, USA) were coated with a reference *O. europaea* extract (1 µg of protein/well) overnight in a humidified chamber. Several dilutions were made from the six varieties. Every dilution was incubated with a serum pool for 2 h at room temperature. A positive and negative control was also included. Afterwards, the dilutions were transferred to the *O. europaea*-coated plates and incubated overnight. After washing, 100 µl of antihuman IgE peroxidase labelled were added and let to stand for 30 min at room temperature. After washing, the plates were developed for 30 min and stopped with H₂SO₄ 1 N. Allergenic activity was evaluated by establishing the 50% of inhibition using as control the pool of serum not inhibited and PBS as negative control.

Estimation of the Ole e 1 content

Two different methods were used to calculate the Ole e 1 content: 1) ELISA inhibition using rabbit specific polyclonal antibodies and 2) scanning densitometry (12). The purification of Ole e 1 and the production of rabbit antibodies were conducted following methods previously described. SDS-PAGE gels of the six varieties were scanned in the Scanner Sharp JX-330 and analyzed with the Image Master 1-D Elite v 2.01 of Pharmacia Biotech.

Results

Skin-test results

The geometric mean of the wheal sizes induced by the six varieties were: Carrasqueño 56.65 mm² (181–12); Manzanillo 54.05 mm² (199–10), Acebuche 42.48 mm² (128–16), Hojiblanco 33.05 mm² (82–10), Picual 58.77 mm² (276–15) and Nevado 51.85 mm² (133–13). The sizes of the wheals were analyzed by the ANOVA test,

confirming significant differences between the varieties of *O. europaea* ($P=0.006$). The Bonferroni test for multiple comparisons, also confirmed significant differences between Hojiblanco and Picual, Carrasqueño, Nevado and Manzanillo and between Picual and Acebuche varieties (Fig. 1).

Protein content

The yields of lyophilized material in the six *O. europaea* varieties were similar. For the variety Carrasqueño the yield was 2.31%; for Manzanillo, 1.84%; for Acebuche, 3.19%; for Hojiblanco, 2.56%; for Picual, 2.38% and for Nevado, 2.27%.

The protein content of the six extracts varied between 994 µg of protein/mg of lyophilized in the Manzanillo extract to 839 µg of protein/mg in the Acebuche extract.

Antigenic and allergenic profile

The analysis of the SDS-PAGE gels of the different olive pollen extracts revealed several proteins a molecular weight range of 5–97 kDa. The most prominent band corresponded to Ole e 1 except in the Acebuche and Hojiblanco varieties (Fig. 2).

The allergenic profile was studied by immunoblot. Several bands were recognized by the serum pool. Ole e 1 and allergens between 30 and 45 kDa were the most prominent molecules recognized in the Carrasqueño, Manzanillo, Nevado and Picual varieties. In the Acebuche and Hojiblanco varieties the most prominent bands corresponded to allergens with a molecular weight between 30 and 45 kDa. Ole e 1 was hardly recognized in the Acebuche variety (Fig. 3). Similar results were obtained using the pool of sera derived from 15 patients from Jaén.

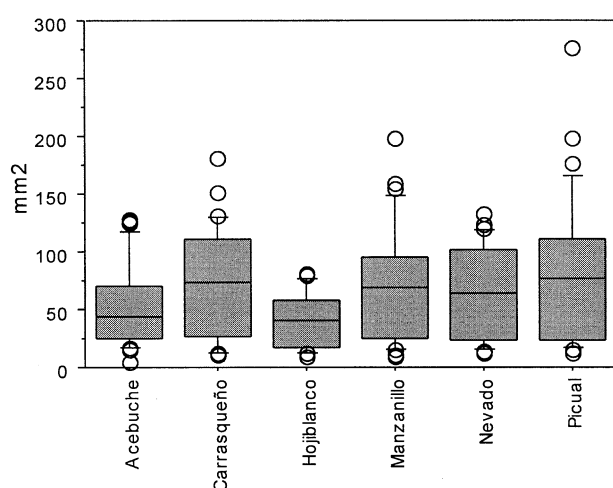


Figure 1 Boxplot view of the wheal sizes induced by the six olive pollen varieties in 29 olive pollen sensitized patients residing in Madrid.

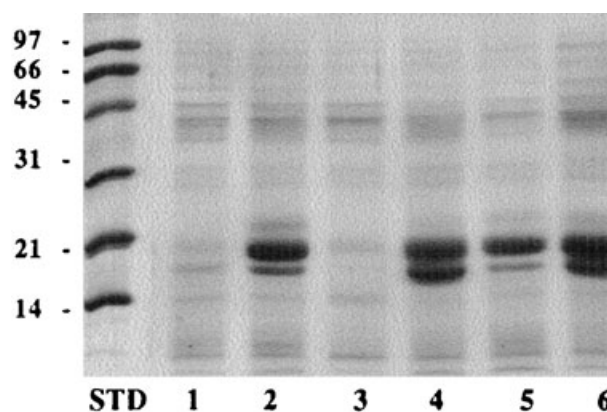


Figure 2 SDS-PAGE. Lane 1, Acebuche; lane 2, Carrasqueño; lane 3, Hojiblanco; lane 4, Manzanillo; lane 5, Nevado; lane 6, Picual.

Biological potency

The study of biological potency, expressed in HEP per g of raw material showed important differences between the varieties. Manzanillo was the variety with the most potency. Acebuche and Hojiblanco varieties had a low potency when compared with the rest of the varieties. Both varieties showed very similar potency. Results are represented in Fig. 4. The statistical analysis of the results demonstrated parallelism of the regression lines of the six extracts ($P=NS$), indicating consistency in the allergen composition of the six extracts. The only differences related to the potency of the extracts.

ELISA inhibition

The six varieties were compared against an *O. europaea* extract by ELISA inhibition. The 50% inhibition value was calculated in all cases. All the curves had a correlation coefficient (r -value) greater than 0.95 and all passed the test for parallelism using the reference *O. europaea* control as the standard. The curves are represented in Fig. 5.

The quantities of protein needed to achieve 50% inhibition were: *O. europaea* standard 0.62 µg, Acebuche 0.50 µg, Carrasqueño 0.82 µg, Hojiblanco 1.01 µg, Manzanillo 0.42 µg, Nevado 0.24 µg and Picual 0.24 µg.

Ole e 1 content

Two different methods were used to determine the total concentration of Ole e 1: ELISA inhibition with rabbit polyclonal antibodies against Ole e 1 and scanning densitometry. The results, expressed in µg of Ole e 1 per µg of whole extract are represented in Table 1. The varieties with the lowest Ole e 1 content were Acebuche and Hojiblanco, whereas Carrasqueño was the variety with the highest content.

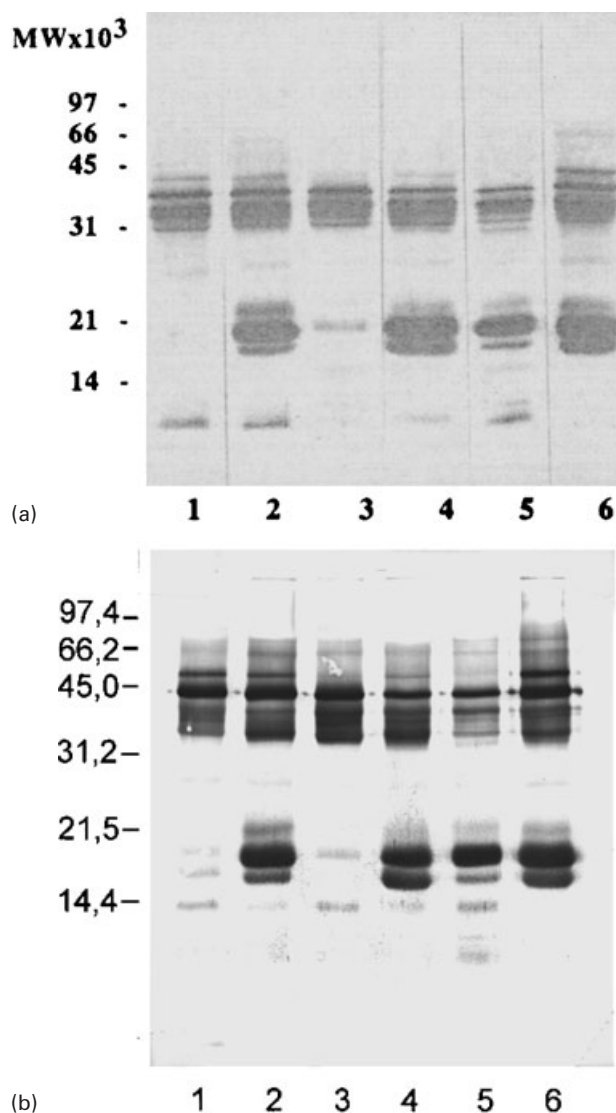


Figure 3 Immunoblot experiments. (a): Pool of sera from patients residing in Madrid. (b): Pool of sera from patients residing in Jaén. Lane 1, Acebuche; lane 2, Carrasqueño; lane 3, Hojiblanco; lane 4, Manzanillo; lane 5, Nevado; lane 6, Picual.

Discussion

The inhalation of olive pollen is one of the most common causes of sensitization and allergic respiratory symptoms in Mediterranean countries (13). In many of these countries, several varieties of olive trees are planted. The selection of the varieties depends on the climatic conditions of the region, the characteristics of the soil and the commercial use of the fruit.

The present study evaluates the allergenic composition of six different varieties of Olive pollen collected in Southern Spain. This study was conceived because of the differences detected in the allergenic composition of olive pollen collected in Spain and in California, US. In this latter study, we confirmed great differences in the antigenic and allergenic composition of 12 batches of olive pollen collected in both countries. Major differences were observed in respect to the content of Ole e 1

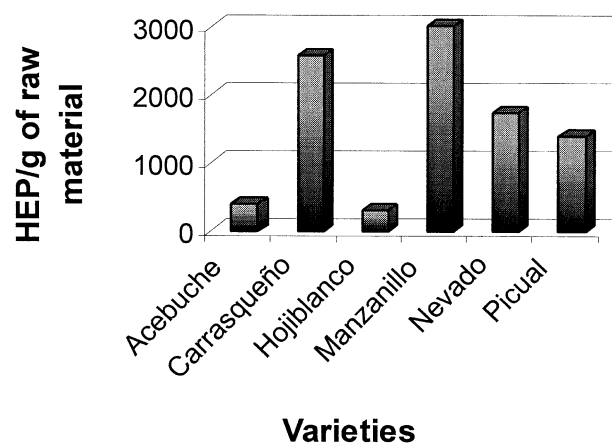


Figure 4 Histogram representing the biological potencies of the six varieties expressed in HEP/g of raw material.

and Ole e 4. In 1984 and 1994, pioneer studies in Spain had already investigated the differences in the allergenic composition of *O. europaea*. These authors evaluated 16 and 12 varieties, respectively, and suggested that the Manzanillo variety was the most allergenic based on skin testing (4, 5). In our study, the Manzanillo variety was also one of the most potent varieties and had the highest Ole e 1 content.

It has been suggested that the allergenicity of the olive pollen could depend of the variety of the olive tree (14, 15). This fact may have important consequences from an allergological point of view. Because different varieties may produce pollen with a different allergenic load, individuals who have an IgE-mediated allergy to olive pollen may experience a stronger or weaker allergic response after the inhalation of olive pollen, depending on the variety of the olive pollen inhaled. It may also have implications when discussing pollen counts in different regions, because some varieties could produce more allergenic pollen than others, thus, needing smaller amounts of pollen to induce sensitization and symptoms. Therefore, exposure to pollen may not be synonymous of antigen exposure. In this case, the immunochemical quantitation of airborne allergens could be a better indicator of allergen exposure.

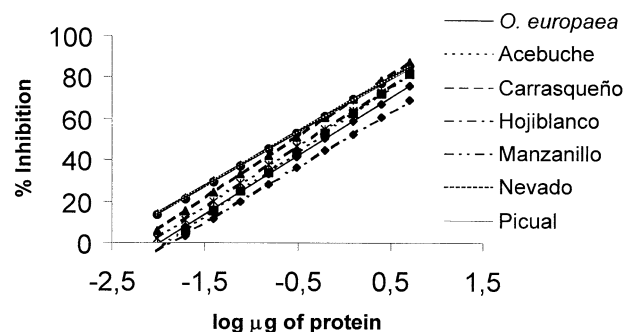


Figure 5 ELISA inhibition experiments to determine the amount of protein needed to obtain 50% inhibition. An internal standard of *O. europaea* was used in the solid phase.

Table 1 Protein and Ole e 1 content, determined by scanning densitometry and ELISA inhibition, of the 6 varieties of *O. europaea*

	Varieties of <i>Olea europaea</i>					
	Acebuché	Carrasqueño	Hojiblanco	Manzanillo	Nevado	Pical
Protein content μg protein/mg lyoph	839.900	970.100	973.400	994.800	993.100	992.100
Inhibition μg Ole e 1/ μg prot	0.076	0.260	0.050	0.232	0.116	0.100
Densitometry μg Ole e 1/ μg prot 0.287	0.612	0.153	0.658	0.677	0.614	

In this study we have confirmed that patients who have an IgE-mediated allergy to olive pollen, exhibit different skin-test responses when tested with pollen of different varieties of the olive tree. This variability could be explained by the differences in the content of Ole e 1 and other important allergens. We acknowledge that these results could be more representative for a population of olive skin-test positive individuals residing in the Madrid area. We have previously demonstrated some differences in the allergic response to olive pollen by individuals from Madrid and Jaén (16). In order to further investigate these previous findings, we used two different serum pools to analyze the allergenic composition of the six varieties by immunoblots in the present study. We have used sera from patients from Madrid, exposed to smaller amounts of olive pollen and from patients from Jaén, who are in contact with larger quantities of pollen. The results demonstrated that the IgE-binding patterns of both serum pools were very similar, suggesting that both populations had been exposed to pollens from the different varieties used in the study.

It has been suggested that the concentration of Ole e 1 depends on environmental and physiological aspects of the tree and not on genetic differences (17). These factors may play an important role in the development of the pollen grain, in the formation of the pollen tube and therefore in the content of Ole e 1 (18). Environmental factors, such as the orientation of the trees in the field and of the flowers from which the pollen grains are collected, or the hours of sun light, may also play an important role to be considered in the future. Other points to be contemplated are the mean temperature of the year, the rainfall in the area and the health status of the tree, since many allergens are pathogenic related proteins (19). All these aspects could influence the development, maturity and final allergenic composition of the pollen grain. Other important aspect to be investigated is if the biology and genetic differences of these olive tree varieties are responsible for the different protein composition and the degrees of allergenicity detected. Castro et al. (20) proposed that Ole e 1 could be used as a molecular marker to distinguish the different varieties. In a recent study, Minelli et al. (21) suggested that the genetic differences in the olive tree varieties could be attributed to polymorphism within chromosomes I, V y VII.

In this study we confirm the complex antigenic profile of *O. europaea* pollen, demonstrating several IgE bind-

ing proteins/peptides. Currently, nine allergens have been identified and published (22). We also evaluated the allergenic potency of the whole extract and not only focussed on a single allergen. The biological potency was measured in the six extracts by ELISA inhibition and by a competitive assay (REINAp), expressed in HEP units. Using both methods, the Hojiblanco and Acebuché varieties had the lowest potency, which was consistent with a lower Ole e 1 content. Ole e 1 has been considered as the major allergen in olive pollen (23, 24). In our study, we observed that the varieties with the lowest concentration of Ole e 1 had less allergenic potency *in vivo* and less IgE-binding capacity *in vitro*. However, all the extracts had a very similar protein concentration, indicating the consistency of the manufacturing process and a uniform composition.

We have used Ole e 1 as a marker to illustrate the changes in the allergenic composition of the olive pollen. Both methods demonstrated that the Hojiblanco variety had the lowest concentration of Ole e 1. By ELISA inhibition, this extract had 0.050 μg of Ole e 1/ μg of protein (5% of the total protein) and by scanning densitometry, 0.153 μg of Ole e 1/ μg of protein (15%). The variety with the highest content was Manzanillo, which had approximately 80% more Ole e 1 than Hojiblanco. However, this variability may not only be restricted to this important allergen and the content of other allergens is also likely to experience shifts. We believe, the variability in the allergenic composition of the different varieties does not have serious implications in the standardization, or manufacturing process of allergen vaccines, since for these purposes, large batches of olive pollen collected from several varieties are used, thus, assuring a complete display of allergens. However, one may argue that a local, or a more complete allergenic variety may be more indicated for immunotherapy.

The existence of less allergenic, or hypoallergenic varieties of olive tree could have a great importance from a prevention and environmental control point of view, since olive trees are currently being planted as ornamental trees in many countries. The introduction of less allergenic trees in gardens and parks therefore seems warranted.

In the near future we will expand this study to include more varieties and conduct a follow-up study of the same varieties over several years in order to verify if the high or low allergenicity detected in this study remains stable.

References

- BOLUDA L, ALONSO C, FERNÁNDEZ-CALDAS E. Purification, characterization and partial sequency of two new allergens of *Olea europaea*. J. Allergy Clin Immunol 1998;**101**:210–216.
- GELLER-BERNSTEIN C, ARAD G, KEYNAN N, LAHOZ C, CARDABA B, WASEL Y. Hypersensitivity to pollen of *Olea europaea* in Israel. Allergy 1996;**51**:356–359.
- BARBER D, CARPIZO J, GARCIA RUMBAO MC, POLO F, JUAN F. Allergenic variability in *Olea* pollen. Ann Allergy 1990;**64**:43–46.
- GONZÁLEZ-QUEVEDO MT., Contribución a la caracterización y estandarización de extractos alergénicos de polen de *Olea europaea* 1984, PhD thesis. Sevilla, España.
- CONDE HERNÁNDEZ M., Aislamiento, caracterización y estandarización del extracto del polen de doce variedades de *Olea europaea* 1994, PhD thesis. Sevilla, España.
- BARRANCO D, RALLO L. Las variedades de Olivo cultivadas en España. Olivae 1985;**9**:16–22.
- BARRANCO D, RALLO L 1984 Las variedades de olivo cultivadas en Andalucía. Madrid, Consejería de Agricultura Y Pesca de la Junta de Andalucía E. Instituto de Estudios Agrarios, . Pesqueros y Alimentarios.
- CLAROS MG, CRISPILLO R, AGUILAR M, CÁNOVAS F. Identificación y clasificación del olivar malagueño a través del ADN. Agromar 1999;**1**:1–8.
- JOHANSSON SGO, HOURIANE J, BOUSQUET J, et al. A revised nomenclature for Allergy. Allergy 2001;**56**:813–824.
- LAEMMLI UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970;**227**:680–685.
- CASANOVAS M, MARAÑÓN F, BEL I. Comparasion of skin prick test assay and reverse immunoassay competition (REINA-C) for biological activity of allergens. Clin Exp Allergy 1994;**24**:134–319.
- BOLUDA L, SASTRE J, CASANOVAS M, FERNÁNDEZ CALDAS E. Determination of Ole e 1 by enzyme immunoassay and scanning densitometry. Validation by skin-prick testing. J Immunol Meth 1999;**223**:17–26.
- WHEELER AW. Hypersensitivity to the allergens of pollen from olive tree (*Olea europaea*). Clin Exp Allergy 1992;**22**:1052–1057.
- BESNARD G, BERVILLE A. Multiple origins for Mediterranean olives (*Olea europaea* L. Ssp. Europaea) based upon mitochondrial DNA polymorphisms. Acad Sci 2000;**323**:173–181.
- WASEL Y, GELLER-BERNSTEIN C, KEYNAN N, ARAD G. Antigenicity of the pollen proteins of various cultivars of *Olea europaea*. Allergy 1996;**51**:819–825.
- CASANOVAS M, FLORIDO F, SAÉNZ E, et al. Sensitization to *Olea europaea*: Geographical differences and discrepancies. Allergol Immunopathol 1997;**25**:159–166.
- LINSKENS HF, VAN DER WERKEN P, JORDE W. The formation of allergens during the development of rye pollen (*Secale cereale*). Allergol Immunopathol 1980;**3**:35–42.
- DE DIOS ALCHE J, CASTRO AJ, OLMEDILLA A, et al. The major olive pollen allergen (Ole e 1) shows both gametophytic and sporophytic expression during anther development, and its synthesis and storage takes place in RER. J Cell Sci 1999;**112**:2501–2509.
- AHLHOLM JU, HELANDER ML, SAVOLAINEN J. Genetic and environmental factors affecting the allergenicity of birch (*Betula pubescens* ssp. Czerepanovii [Orl.] Hamet-Ahti) pollen. Clin Exp Allergy 1998;**28**:1348–1388.
- CASTRO AJ, ALCHE JD, CUEVAS J, RODRIGUEZ GARCIA MI., Implicaciones del olivo en el medio ambiente. Proteínas alergénicas del pólen de olivo como herramienta de selección de variedades del olivar con fines ornamentales. IX. Simposio Científico-Técnico Expoliva 99. Jaén. Junio 99.
- MINELLI S, MAGGINI F, GELATI MT, ANGIOLILLO A, CIONINI PG. The chromosome complement of *Olea europaea* L. characterization by differential staining of the chromatin and in situ hybridization of highly repeated DNA sequences. Chromosome Res 2000;**8**:615–619.
- ALLERGEN NOMENCLATURE SUBCOMMITTEE., Official list of allergens. URL: <http://www.allergen.org>.
- LAHOZ C, LAZURICA P, TRICAS L. A glycoprotein is a major antigen in *Olea europaea*. Ann Allergy 1985;**55**:289.
- LAZURICA P, MARURI N, GALOCHA B. Olive (*Olea europaea*) pollen allergens. Isolation and characterization of two major antigens. Mol Immunol 1988;**25**:337–344.