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Detection of airborne allergen (*Ole e 1*) in relation to *Olea europaea* pollen in S Spain

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Clinical and Experimental Allergy

Summary

Background In recent years, it has been demonstrated that the air carries not only airborne pollen but also plant particles of smaller size that have allergenic activity, and, being within the respirable range, these particles can trigger rapid attacks in the lower respiratory tract. The study of particles according to size (0.7–40 μm) could provide valuable information on the real allergenic activity in the atmosphere.

Objective The purpose of this study was to analyse the dynamics of airborne *Olea europaea* pollen in contrast to the allergenic activity of *Ole e 1* in the atmosphere.

Methods The analyses were carried out with a Hirst-type volumetric collector and a cascade impactor simultaneously during the MPS of the olive. The indirect ELISA was used to detect the allergenic activity. The sampling was performed in Granada city centre (S Spain), in the Science Faculty building on the University of Granada from 30 April to 26 June 2005.

Results and conclusions This research demonstrates that both the allergenic activity as well as the pollen particles follow in a similar curve, except in periods before or succeeding the main *Olea* pollen season. The study of the distribution of the allergenic particles according to their sizes reveals that the highest concentrations are between 3.3 and < 0.7 μm, thus indicating that allergenic activity primarily involves paucimicronic particles.

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Introduction

During the last few decades, respiratory allergopathies have significantly increased. These diseases, in particular those related to pollen allergy, are more frequently reported in industrialized countries, leading to a general consensus that the rate of respiratory ailments is higher in industrialized areas than among people living in rural areas [1, 2].

A quarter of all solid elements dispersed in the air are biological in origin [3], and numerous studies report that the concentrations of these types of particles are progressively increasing [4]. This intensification is being noted mainly in the Mediterranean area, where, according to specialists [5, 6], the pollen of anemogamous plants occupy one of the first positions in the biological spectrum of the air. This phenomenon is triggering major complications in public health, as respiratory maladies have now reached an estimated 20% of the population in industrial societies [7], making air control necessary for improve the health and quality of life in allergy patients.

Most asthma crises appear to be provoked by pollen grains [8–10], although recent studies affirm that the solid particles larger than 20 μm are incapable of penetrating to the lower respiratory tract [11, 12], and the average pollen size ranges from 15 to 40 μm. One of the theories to explain this apparent contradiction is that the pollen grain, on making contact with the mucosa, releases allergens that could rapidly penetrate to the bronchia, provoking asthmatic symptoms [13]. Moreover, it has been recently demonstrated that the air, in addition to carrying airborne pollen, contains allergenically active plant particles of smaller size, being submicronic (< 10 μm) and paucimicronic (< 1 μm). These particles, being within the respirable fraction, can penetrate through the nasal passages to the bronchia, quickly provoking allergic symptoms [12, 14, 15].

The study of these particles, according to their size, could provide ample information on the real allergenic activity in the atmosphere. This line of research has been followed previously on certain fungal taxa and vascular plants, such as *Ambrosia* [16–18], *Quercus* [19], *Poaceae* [14, 20, 21], *Betulaceae* [15, 20, 22, 23] and *Alternaria* [18, 24], indicating that in the submicronic and paucimicronic fractions, there is a heavy allergenic load. Each of these studies was performed with some variations in the detection method, although, in a general way, measurement tests of antigenic activity were performed by ELISA or RAST, using specific antibodies for each of the taxa analysed.

Olea europaea pollen is the major cause of allergy in the Mediterranean area [25], the second cause of pollinosis in Spain after *Poaceae* pollen [26]. This is also the primary cause of pollinosis in the province of Granada [27] (S Spain), given that 79.5% of the patients studied had this type of allergy. The present work delves into the study of olive pollen from an aerobiological and allergological standpoint.

At present, 10 allergens have been identified and characterized in *Olea* pollen. One, *Ole e 1*, a protein recognized as a major allergen, with a PM of 20 kDa, represents up to 20% of the protein content in pollen [28]. *Ole e 1* is a specific allergen that is detected only in pollen tissue and not in leaf, fruit or stem tissues [29]. Immunolocation assays determine that it is stored mainly in the endoplasmic reticulum [30], while at mature stages of the grains this protein can also be found in the exine, where it could be involved in recognition between the pollen and stigma or between the pollen tube and stile cells [31]. Furthermore, *Ole e 1* plays an important role in the hydration of the pollen grain, and hence is found in high concentrations [30]. These observations help explain the fact that this protein affects more than 70% of the patients [31] sensitive to *Olea*.

Using olive pollen as the prototype, we present an innovative technique, the indirect ELISA, which has been perfected with reaction times appreciably reduced for a quicker and more effective analysis of allergen activity in the air. In addition, the results found by this test are compared with the aerobiological behaviour of olive pollen in the atmosphere of the city of Granada (S Spain), characterized by the high levels reached by this type of pollen during spring.

Materials and methods

Technique for capturing airborne antigenic particles

The air was sampled in the pollination period of *Olea europaea*, from 30 April to 26 June 2005, using a cascade impactor collector (Andersen Inc.; Atlanta, GA, USA) situated on the terrace of the Science Faculty (University of Granada) at a 23 m height in Granada city centre (S Spain). This collector was placed during the middle hours of the day, as the continental climate in this province causes higher temperatures between 12 and 17 h, with a consequent increase in olive-pollen concentrations [32]. This collector is used to determine the distribution of sizes and total concentrations of all the solid particles or liquids suspended in the air. The air flow through the impactor is controlled by a pump that draws in air at 30 L/min (Lanzoni SPS 3001, Bologna, Italy). The size discrimination of the particles is possible by the variation in air velocity, which is led sequentially through a series of Whatman® fibreglass filters (Glass microfibre filters; type: GF/A, 1.6 μm, Whatman, Kent, UK). These filters, recommended for gravimetric determination of airborne particulates, stack sampling and absorption methods of air-pollution monitoring, offer fine-particle retention and a high flow rate, as well as good loading capacity. The largest particles are deposited in the first stages while the smallest pass through the collector until being stopped by the correspondingly fine filter. Stage 0 (the first stage) collects particles having aerodynamic diameters greater than 9 μm; stage 1 corresponds to > 9–5.8 μm; stage 2 to 5.8–4.7 μm; stage 3 to 4.7–3.3 μm; stage 4 to 3.3–2.1 μm; stage 5 to 2.1–1.1 μm; stage 6 to1.1–0.7; and stage F to < 0.7 μm. The final stage contains the remains of particles not collected by foregoing stages. In this way, the collector reproduces the collection characteristics of the pollen in the human respiratory tract, and therefore the particle distribution in stages serves as an indicator of the degree of penetration of the particles in the respiratory system.

Techniques for quantifying airborne antigenic activity and standard curve: indirect ELISA

The filters, removed daily and stored cold (4 °C) for a maximum period of 20 days, were analysed in the following way. For each filter, four circular replicates (Ø = 0.5 cm) were taken on a radial pattern. For control, four replicates of one circular filter with no impact were used. All the filters were submerged in 125 μL phosphate-buffered saline (PBS, pH 7.4) in microplate wells for 15 h at room temperature and saturated atmospheric humidity, thereby permitting the release of the *Olea* pollen antigens, which then adhered to the walls of the microplates. The discs were removed and the wells cleaned with 200 μL PBS–TW (0.3% Tween 20), to which 200 μL PBS–TW-Gelatin from porcine skin (0.3% Tween 20+1% Gelatin from porcine skin, FLUKA®, Buchs, Germany) were added and incubated for 1 h at 37 °C; this blocking solution covers the walls of the microplates where there are no antigens. Afterwards, the wells were washed again with 200 μL PBS–TW (0.3% Tween 20).

The *Olea europaea* antigens were detected by adding 125 μL of antibodies produced by rabbit (*Ole e 1* rabbit, Bial-Aristegui S.A., Bilbao, Spain. Natural allergen from *Olea europaea* pollen extract purified by standard chromatography [33], purity > 98%; binds with specific human IgE in ELISA and Western blot) diluted to 1 : 1000 in PBS and incubated 45 min at 37 °C. Next the wells were cleaned with 200 μL PBS–TW (0.3% Tween 20), later adding 125 μL HRP (Polyclonal Swine Anti-Rabbit Immunoglobulins, Dako Cytomation®, Glostrup, Denmark) diluted in PBS at a concentration of 1 : 1000. This mixture was incubated 30 min at 37 °C and afterwards washed with 200 μL PBS–TW (0.3% Tween 20). The enzyme activity of the bound anti-rabbit IgG–HRP conjugate was determined by adding 125 μL *O*-Phenylenediamine tablets (Sigma®, Steinheim, Germany) diluted in citrate buffer (1 tablet of OPD + 12.5 mL buffer citrate + 12.5 mL distilled water + 20 μL H_2O_2) and incubated for 20 min at room temperature in the absence of light. This reaction was stopped by adding 50 μL of HCl 3 N. The results were measured with a microplate reader (Multiskan EX, Thermo Labsystems, Madrid, Spain) capable of reading at 405 nm.

The pattern curve of *Olea europaea* was drawn from dilutions with carbonate buffer (pH: 8.5) of purified protein (*Ole e 1*) (Bial-Aristegui S.A., Bilbao, Spain) at concentrations of 0.9765 125 ng/mL distributed regularly throughout the columns of the microplates. Next, the protocol explained above was followed again. The curve was adjusted using the mathematical software *Table Curve 2D V. 5.1.*

Airborne-pollen sampling

The aerobiological samples were collected continuously during 2005 with a volumetric collector, the Burkard Spore Trap [34]. The counting method was that recommended by the Spanish Aerobiological Network (REA) [35]. The daily pollen data are expressed in grains per cubic metre (grains/m^3). The two collectors, adjacent, functioned simultaneously for a reliable comparison of the results of the two samples.

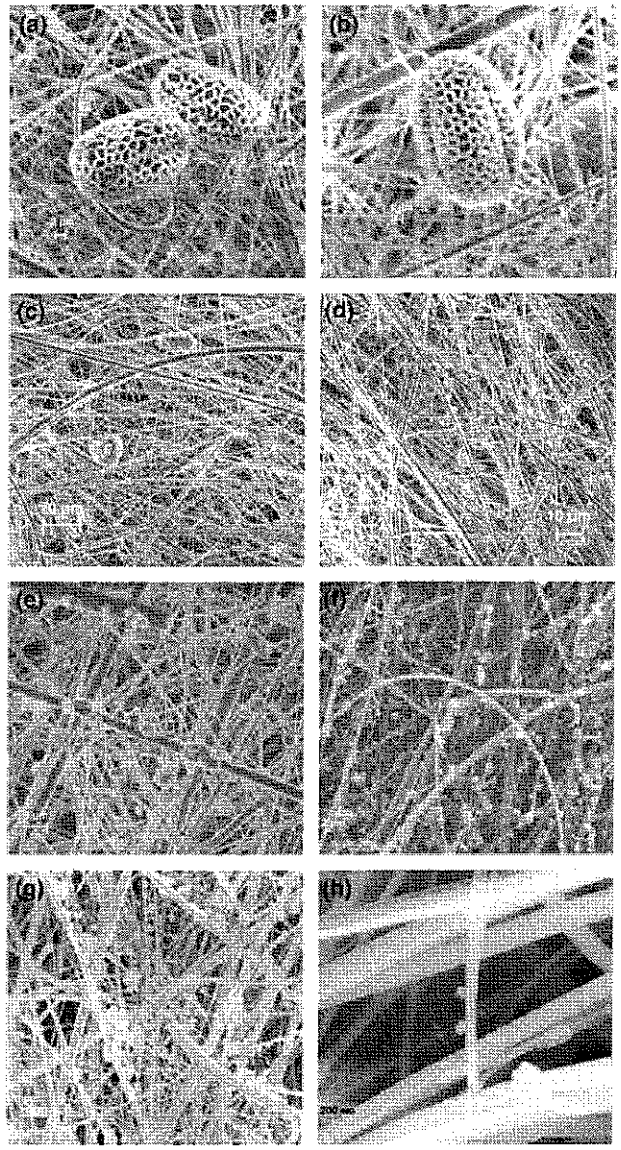

Fig. 1. Scanning electron microphotographs of air filter of stages. (a) > 9 μm (stage 0), (b) > 9–5.8 μm (stage 1), (c) 5.8–4.7 μm (stage 2), (d) 4.7–3.3 μm (stage 3), (e) 3.3–2.1 μm (stage 4), (f) 2.1–1.1 μm (stage 5), (g) 1.1–0.7 μm (stage 6) and (h) < 0.7 μm (stage F).

Results

Sizing of airborne particles

After the peak day (19 May 2005; i.e. the highest detected concentration of *Olea* airborne-pollen), the exposed filters were examined using a scanning electron microscope (LEO 1430-VP, Leo, Oberkogen, Germany) in order to verify the sizing efficiency of the Andersen cascade impactor (Fig. 1). We found pollen grains only in stages 0 and 1. The rest of the stages were pollen free. We confirmed the sizing efficiency of the other stages of Andersen cascade impactor as indicated by the manufacturer.

Standard curve

The purified protein (*Ole e 1*) is especially hydrophobic. To make the aforementioned dilutions, we need to dilute the protein in a homogeneous way. This was achieved only at pH >8.5. On the contrary, the daily sample did not require the use of this buffer, as in this case it was not necessary for the antigens to be distributed uniformly on the walls of the wells. The rate of colour formation in ELISA was proportional to the amount of antigen, being independent of the aggregation state of the antigen.

Table 1. Results for the standard curve of *Ole e 1*

Variables in the equation	Equation	R^2	ANOVA Source	Sum of squares	DF	Mean square	F	P
a = 1.96E-03			Regression	0.003	1	0.002		
	$y=a+bx^{(0.5)}$	0.992	Error index	0.000	4	0.000	539.349	0 000
b = 5.21E-03			Total	0.003	5			

The Table 1 shows the fit of the pattern curve to the equation whose coefficients as well as the regression index (r^2) and ANOVA parameters are reflected.

Airborne pollen/antigens

As in previous years, the trend of the *Olea* pollen in 2005 was characterized by marked seasonality, reaching very high concentrations in a relatively short period. The heaviest pollen began to be detected at the beginning of May until early June. During this period, a total of 24 188 *Olea* pollen grains were collected, a number very similar to that detected in previous pollen seasons. Both the phenological behaviour as well as the pollen production of these trees in 2005 followed patterns similar to those of other years, and thus the data used in the present study were within the standard ranges of pollen concentrations.

The continuous atmospheric sampling of both the allergenic activity as well as the pollen particles were quite parallel, mainly in the period from 28 May to 1 June, when the airborne pollen increases or decreases in a manner similar to that of the allergenic activity (Fig. 2). The pollen and allergen concentrations reached their highest values on the same date (29 May), with 2648 pollen grains/m^3 and 1704.8 ng of allergens/m^3. Although less similar, the concentration patterns of both particles were comparable during the second and the third week of the same month (7–16 May; 20–23 May). By contrast, during the periods preceding and succeeding the heaviest *Olea* pollination (MPS), two curves showed notable divergence.

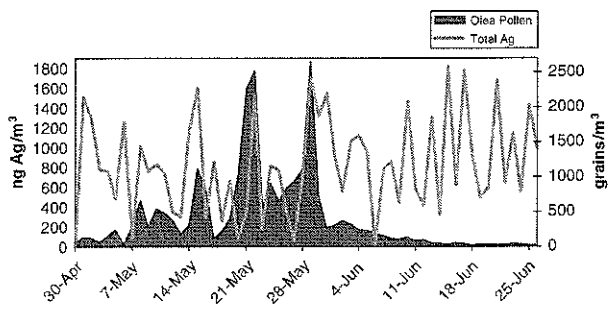

Fig. 2. Comparison between antigenic activities of *Olea* pollen in air samplers and daily fluctuations in the airborne *Olea* pollen counts per cubic metre of air from April to June.

Airborne pollen/antigenic stages of distribution

One of the objectives of this analysis was to determine the distribution of the allergen particles according to their sizes at the different stages. As reflected in Fig. 3 and Table 2, the allergen load was arranged according to the different particle sizes. It is especially noteworthy that the highest concentrations of allergens, for which the size was within the respirable fraction, were located between the stages 4 and F (3.3 to < 0.7 μm). Only 6.49% of the total allergenic activity corresponded to stages 0 and 1, where the pollen grains struck. In the same way, Table 3 shows the allergenic activities of *Olea* pollen in different particle-size fractions during: MPS, before MPS and after MPS. The same behaviour was found during the three periods.

Discussion

This study investigates the relationship between the pollen concentrations and allergen activity in the atmosphere of a highly allergenic plant species, *Olea europaea*. It has been adequately demonstrated that there is a similar aerobiological dynamic between the two variables, especially during the development of the MPS *of Olea* (Fig. 2).

The confirmation of allergenic activity *Ole e 1* proved highly significant during the periods before and following pollination, as reported by Pehkonen and Ratio-Lemtimaki [15], Spieksma et al. [14] and Schäppi et al. [21] for other plant species. These authors note that there may be an allergenic load by other parts of the plant, such as buds, aments and bark [15, 36], or that there may be a resuspension of such particles after pollen deposition on the ground [22]. Other studies examine the possibility that the sampling may give rise to a crossed reaction with other pollen types [15].

Although we do not rule out any of the above hypothesis, the last two could explain the allergenic activity detected in the periods preceding and succeeding *Olea* MPS, as *Ole e 1* is the specific allergen pollen and is not found in buds, aments or bark [29]. It has been demonstrated that there is a very severe crossed reaction between the species making up the family *Oleaceae* [26, 37, 38], including privet (*Ligustrum* sp.), ornamental shrubs and trees, which are used considerably in Granada and that pollinate in the dry period (June-July). This pollen is characteristically very heavy and therefore rarely appears

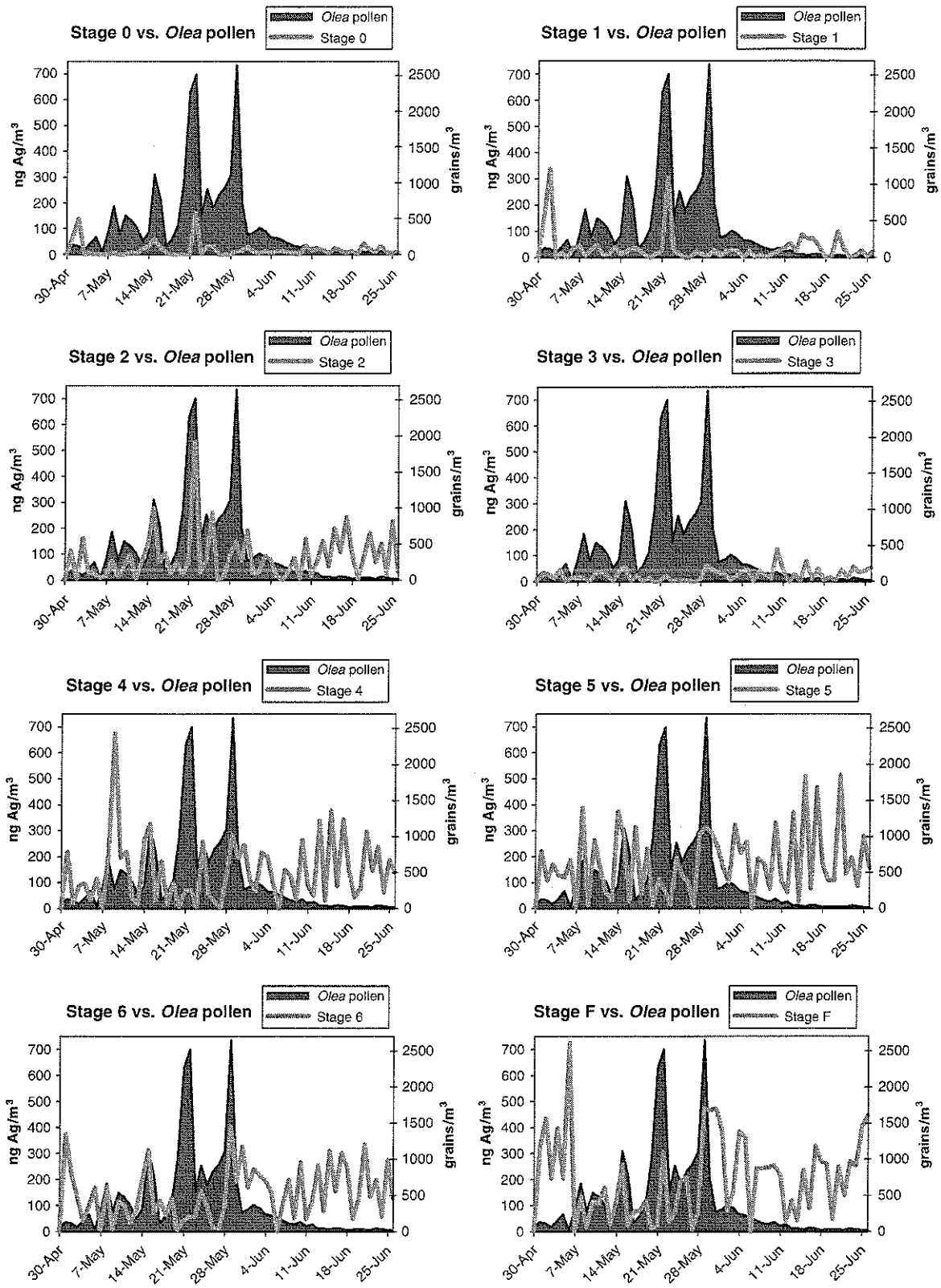

Fig. 3. Comparison between relative *Olea* pollen allergenic activities in different particle-size fractions vs. daily fluctuations in the airborne *Olea* pollen counts per cubic metre of air from April to June > 9 μm (stage 0), > 9–5.8 μm (stage 1), 5.8–4.7 μm (stage 2), 4.7–3.3 μm (stage 3), 3.3–2.1 μm (stage 4), 2.1–1.1 μm (stage 5), 1.1–0.7 μm (stage 6) and < 0.7 μm (stage F).

Table 2. Allergenic activities of *Olea* pollen in different particle-size fractions, expressed as percentages and total sum allergens of indirect ELISA

Stages	Impaction stages (μm)	Impaction stages (%)	Total allergens (ng/m^3)
0	> 9.0	2.12	1016.77
1	> 9.0–5.8	4.37	2094.89
2	5.8–4.7	10.64	5098.32
3	4.7–3.3	2.92	1400.43
4	3.3–2.1	17.00	8146.80
5	2.1–1.1	21.16	10142.92
6	1.1–0.7	17.42	8347.42
F	< 0.7	24.37	11676.94
Total sum			47924.49

Table 3. Allergenic activities of *Olea* pollen in different particle-size fractions during MPS, before MPS and after MPS, expressed as percentages of indirect ELISA and total sum allergens

		Period before MPS		MPS		Period following to MPS	
Stages	Impaction stages (μm)	Impaction stages (%)	Total allergens (ng/m^3)	Impaction stages (%)	Total allergens (ng/m^3)	Impaction stages (%)	Total allergens (ng/m^3)
0	> 9.0	3.4	213.59	2.1	554.34	1.6	248.81
1	> 9.0–5.8	9.7	600.25	3.4	903.79	3.8	590.84
2	5.8–4.7	5.8	357.25	11.5	3036.47	11.1	1704.58
3	4.7–3.3	2.3	143.93	2.9	775.55	3.1	480.94
4	3.3–2.1	9.3	547.07	18.6	4906.79	17.4	2665.93
5	2.1–1.1	15.3	943.86	22.0	5815.46	22.1	3383.59
6	1.1–0.7	16.9	1045.38	16.9	4483.38	18.4	2818.63
F	< 0.7	37.2	2297.13	22.5	5959.52	22.3	3420.28
Total sum			6175.50		26435.34		15313.63

in pollen counts [39]. In Granada, a total of 22 grains/m^3 were measured during the study year. *Lig v 1*, which has a 145-residue polypeptide chain with an N-glycosylation site at Asn-111 and six Cys residues, presented a high degree of similarity to *Ole e 1* isoforms (85–95% identity) [40, 41].

When this pollen falls to the soil, it could fragment, releasing its protein load, and this atmospheric phenomena could be resuspended and be captured by the *cascade impactor* [18]. As reflected in Fig. 3 and Table 3, the allergenic activity after the *Olea* flowering period begins at stage 2 and therefore in paucimicronic fractions, implying resuspension.

The allergenic activity during the period before the heaviest pollination of olive is also greater than that of airborne pollen. Recent research [26] shows that *Ole e 1* has a certain homology with allergens of *Plantago* (*Pla l 1*), *Lolium* (*Lol p 11*) and *Chenopodium* (*Che a 1*).

The N-terminal amino acid sequence determined for *Pla l 1* showed a partial identity (30%–40%) with *Ole e 1*. It has six Cys residues, a potential site of N-glycosylation and a homology with *Ole e 1*, depending on the isoform considered [26].

A comparative study between the aerobiological behaviour of these taxa and the allergenic load of *Ole e 1* revealed that *Plantago* registered its highest concentrations of the year between 1 and 5 May, an increase correlated with the first peak of *Ole e 1*. This could account for the high allergenic activity detected in the period before the *Olea* season. Also, this fact is corroborated by the distribution of the allergenic activity reflected by all the stages of the collector, with high levels appearing at stage 0 and 1, where the pollen grains are found (Fig. 3).

Other possible causes would be the dispersion, on a medium scale, of paucimicronic particles from cultivation in Jaén with earlier flowering [42] and the other could be that some of the particles collected by the cascade impactor could fracture on impact and release their allergens, these being carried to lower stages, although broken pollens have not been observed in the scanning electron microscope.

Also, with the present work, thanks to the characteristics of the collector used, the discrimination of particles by sizes has enabled us to determine the location of the greatest allergen, *Ole e 1*, and thus we can state that it is located both in the respirable as well as the non-respirable

fraction (Fig. 3 and Tables 2 and 3). Nevertheless, the greatest concentrations are found in the paucimicronic fractions, explaining the high percentages of asthmatic symptoms in the allergic population.

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