Immunochemical and physical quantitation of grass and olive pollen allergens and correlation with asthma admitions in Cáceres, Spain

Allergen and pollen counts as markers/predictors of allergic asthma

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ABSTRACT

Background: The association between pollen counts and allergen levels in the air is controversial.

Objective: The aim of the study was to quantify total and major allergen levels of *Phleum pratense* and *Olea europaea*, and to analyze their correlation with grass and olive pollen counts and the number of asthmatic crisis attended in the Hospital Complex of Cáceres, Spain.

Material and methods: A volumetric air sampler and a Burkard spore-trap were used for pollen and aeroallergen collection during April-June 2011. Filters were extracted and major allergens were quantified by ELISA.

Results: May was the main grass pollination period, showing a maximum peak of 1,362 grass pollen grains/m^3 (May 13th). The main pollination period of olive was April 30th-May 20th, showing a maximum peak of 851 olive pollen grains/m^3 (May 11th). Moderate correlation between grass pollen counts or *Phleum* total allergen levels and asthma exacerbations were observed, which increased when a 3-day offset was introduced. Significant association between asthma exacerbations and levels of olive total allergens or olive pollen grains was observed when a 1-day offset was introduced. The maximum correlation (moderate-high) was observed 4 days and 6 days away from the maximum olive pollen peak and the maximum Ole e 1 peak level, respectively.

Conclusions: This study reveals a significant correlation between grass and olive pollination and the increase of the number of visits to the emergency room due to asthma crisis. The aerobiological pattern of allergen levels in the air is comparable to the pollen counts during the grass and olive pollination periods.

Key words: Grass, Olive, Pollen, Allergens, Levels, Phl p 1, Phl p 5, Ole e 1

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RESUMEN

Antecedentes: La relación entre los niveles de pólenes en el aire y los niveles de alérgenos es controvertida.

Objetivo: El objetivo de este estudio fue cuantificar los niveles de alérgenos principales y totales de *Phleum pratense* y *Olea europaea*, y analizar su relación con los niveles de pólenes y el número de crisis asmáticas atendidas en el Complejo Hospitalario de Cáceres, España.

Material y métodos: Se captaron pólenes y aeroalérgenos durante Abril-Junio de 2011, utilizando un colector de aire volumétrico y una trampa Burkard. Se extrajeron los alérgenos de los filtros y se cuantificaron mediante ELISA.

Resultados: Mayo fue el periodo de mayor polinización, (13 de Mayo, pico máximo de 1.362 granos de polen de gramíneas/m^3). El mayor periodo de polinización del olivo fue del 30 de Abril al 20 de Mayo, (11 de Mayo, pico máximo: 851 granos de polen de olivo/m^3). Se observó una correlación moderada entre los niveles de polen o alérgenos totales y exacerbaciones de asma, que aumentó al introducir un desfase de 3 días (*Phleum*), y de 1 día (olivo). La máxima correlación se observó a los 4 y 6 días del pico máximo de polen y de Ole e 1, respectivamente.

Conclusiones: Este estudio muestra una correlación significativa entre la polinización de gramíneas y olivo y el aumento del número de visitas a urgencias debidas a crisis asmáticas. Los patrones aerobiológicos de los niveles de alérgenos en el aire son comparables a los recuentos de pólenes durante los periodos de polinización de gramíneas y olivo.

Palabras clave: Gramíneas, Olivo, Polen, Alérgenos, Niveles, Phl p 1, Phl p 5, Ole e 1

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BACKGROUND

The presence of pollen allergens in environmental air samples have been widely studied. These studies, focused in the detection of pollen from grass [1-3], olive [4-6], Urticaeae [7, 8], Betulaceae [9], ragweed [10, 11], *Plantago* spp. [12], *Platanus* spp. [13] and Cupresaceae [14, 15]. Pollen allergens have been detected in leaves or stems, orbits and pollen starch granules [7, 16, 17] which may contribute to the total allergen load. Allergens are released from pollen grains after water exposure, as submicronic particles that leave the grain directly through the pollen pore (grasses), through a pollen tube (Betulaceae), or by exudation through the exine layer (Urticaceae) [18-20]. Some external factors, as air pollution (diesel particles), can favor allergen release or act as vehicles of the allergens [21, 22].

Association between pollen counts and allergen levels in the air is controversial. While several authors have shown a low correlation coefficient between pollen and allergen levels, other have revealed the presence of the allergens in the environment before and after the pollination period [1-3, 10, 11].

Most of the allergens have molecular weights of less than 100 kDa, and they can easily enter the respiratory airway [3, 6]. The exposure to allergens can be measured by quantification of the pollen allergens in the air [3, 6]. Some studies have shown a better correlation between allergic symptoms in patients sensitized to pollens and pollen allergen levels, than with the number of pollen grains [2, 4, 14].

The aim of the present study was to quantify total allergen and major allergen levels in the air of Caceres, Spain during the spring of 2011 and to analyze their correlation with 1) grass and olive pollen counts, and 2) number of asthmatic crisis evaluated at the Hospital Complex of Cáceres during this time period.

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MATERIALS AND METHODS

Pollen Counts

Pollen grain counts were measured using a Burkard spore-trap (Burkard Manufacturing Co., Rickmansworth, Herts, UK) placed at a height of 15 m in Caceres during the sampling period (April 11th to June 30th, 2011). The air flow rate was 10 L/min. Pollens were caught on 24-mm wide transparent tape coated with a thin film of petroleum jelly, mounted on a cylinder rotating at a speed of 2 mm/h. The adhesive tape (samples) was changed each Monday and divided into seven parts, one for each day of the week. Samples were prepared according to the usual technique, and they were examined under optic microscope with at 40x objective lens. The pollen concentrations were expressed as pollen grains/m^3 of air.

Air Sampling

A volumetric air sampler (Air Sentinel, Quan-Tec-Air Inc., Rochester, Minnesota, USA) was used for aeroallergen collection and run continuously during the same period described above. The sampler was in the same location as the Burkard spore-trap. The air flow rate was 10 m^3/h. Airborne particles were collected by polytetrafluoroethylene (PTFE) filters (Quan-Tec-Air Inc.), which are 99.9% efficient at 0.3 μm. The sampling time for each filter was 24 hours, that is, 240 m^3 of air per sample. Filters were replaced at approximately the same time each day. After extraction, the filters were sealed in plastic bags and stored at 4ºC.

Filter Extraction and Allergen Quantification

A total of 111 PTFE filter membranes were collected and individually extracted in tubes containing 0.5 ml of 0.01M phosphate buffered saline (PBS). Each tube was stirred until the filter was completely wet and incubated overnight in a Rotary mixer (Labinco B.V., Breda, The Netherlands) at 4ºC. Samples were then centrifuged and the supernatant was collected and processed immediately to avoid loss of allergenic activity. Total allergenic activity and allergen content were measured in eluted samples using validated enzyme-linked immunosorbent assays. Total *Phleum* and olive pollen allergenic activity were measured using In house reference

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preparations and an ELISA system. Phl p 1**,** Phl p 5**,** Ole e 1 (Bial Aristegui, Bilbao, Spain) were measured using the manufacturer's instructions. Positive and negative controls were also included. The percentage inhibition was calculated using the corresponding inhibition curve determined from the analyzed pollen. Allergen concentrations were extrapolated using the standard curve and were based on the inhibition capacity; the final results were expressed in micrograms of allergen per milliliter.

Emergency department visit data

The Hospital Complex of Cáceres covers an area of approximately 10,500 m2 and 196,000 inhabitants in southwestern Spain and consists of 2 hospitals in the center of the city. A record was made of all the visits to the emergency room for asthma during the months of April to June 2011. The computer record of the admission department of the emergency department was reviewed. Subsequently, the emergency department files and the medical reports of the cases were reviewed to select those compatible with an asthmatic exacerbation. The study was approved by the ethics committee of the hospital.

Statistical Analysis

Correlations between pollen grain concentrations, aeroallergen levels and asthma exacerbations were analyzed using the GraphPad Prism software for Windows. The Pearson rank correlation and P values were obtained for each analysis.

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RESULTS

Pollen counts

Grass pollen levels quantified for the whole year of 2011 (12.561 pollen grains/m^3) represented 29% of the total pollen grain levels. May was the main grass pollination period (10.579 grass pollen grains/m^3), showing a maximum peak of 1.362 grass pollen grains/m^3 in May 13th.

Levels of olive pollen grains in 2011 (6.998 pollen grains/m^3) supposed a 16.18% of the total pollen grain levels. The main olive pollination period was from April 30th to May 20th (5.892 olive pollen grains/m^3), showing a maximum peak of 851 pollen grains/m^3 in May 11th.

Immunochemical quantification of aeroallergens

The highest levels of total grass allergen activity, as measured with the *P. pratense* immunoassay, were detected between the 4th and the 25th of May. The maximum peak was detected in May 11th (*P. pratense* total allergen level: 145 ng/m^3; Phl p 1 level: 3 ng/m^3; Phl p 5 level: 1 ng/m^3).

The highest levels of total olive allergen were detected between the 5th and the 25th of May. The maximum peaks for olive allergens were detected in May 6th (olive total allergen level: 153 ng/m^3; Ole e 1 level: 11 ng/m^3) and May 9 (olive total allergen level: 104 ng/m^3; Ole e 1 level: 19 ng/m^3).

Asthma exacerbations

From April to June, 111 visits to the emergency area due to asthma exacerbations were registered (18 in April, 80 in May and 13 in June). 22 of the patients (ages from 5 to 88, mean age 37) required of hospital admission, 15 of them during May.

Correlation among pollen counts, aeroallergen concentration and asthma exacerbations

A positive correlation was observed between *P. pratense* total allergen levels and the Phl p 1 and Phl p 5 major allergens levels: *P. pratense vs*. Phl p 1 $r = 0.78$, $p < 0.0001$; *P. pratense vs*. Phl p 5 $r = 0.85$, $p < 0.0001$ (Figure 1A). A moderate positive correlation between grass pollen

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grain counts and allergen levels was observed: grass pollen grains *vs*. *P. pratense* allergens r = 0.57, p < 0.0001; grass pollen grains *vs*. Phl p 1 r = 0.45, p < 0.0001; grass pollen *vs*. Phl p 5 r = 0.58, p < 0.0001 (Figure 1B). In May 13th, the highest pollen levels and the lowest allergen levels were observed may due to the presence of grass pollens from species such as *Cynodon dactylon* which do not fully cross-react with *P. pratense*.

Positive correlations were observed between *O. europaea* total allergen levels and Ole e 1 allergen content: r = 0.88, p < 0.0001 (Figure 2A), and between olive pollen grains *vs*. olive allergens: r = 0.71, p < 0.0001 (Figure 2B) and olive pollen grains *vs*. Ole e 1: r = 0.57, p < 0.0001.

Table 1 shows the correlation results observed between visits to the emergency room due to asthma exacerbations and the levels of the different allergens. Correlations between grass pollen counts or *Phleum* total allergen levels and asthma exacerbations were observed. When a 3-day offset is introduced, the correlation increases to a moderate range.

Significant association between asthma exacerbations and levels of olive allergens or olive pollen grains was also observed when a 1-day offset is introduced. The maximum correlation (moderate-high) was observed 4 days away from the maximum olive pollen peak and up to 6 days after the maximum Ole e 1 peak level.

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DISCUSSION

Grass and olive tree suppose two of the most important sources of pollen in Spain. The Cáceres aerobiological station has been working since 2008 and pollen measurements made during 2008-2016 (unpublished data), revealed that grass pollen is one of the most prevalent pollens detected in the air (mean annual values, 8.237 grains/m^3), followed by *Quercus spp.* pollen (8.462 grains/ m^3). These values suppose a 25% of the total pollen grains counts/m^3/year. Grass pollination period mainly occurs in May, between weeks 17 and 22, with peaks of >1.000 grains/m^3. Olive tree constitutes the third pollen taxon in importance in Cáceres, depending on the year, with a main pollination period between weeks 18 and 21 and with average levels of 4.124 grains/m^3/year.

Most of the patients from this region are sensitized to pollens and show signs and symptoms of rhinoconjunctivis and asthma with a higher frequency and intensity during May, when the grass and olive pollens are predominant. The results obtained in this study reveal the importance of 2011, in terms of grass and olive pollen counts. The pollen levels obtained during this period were above the average, showing a grass pollen peak of 1.362 grains/m^3 in May 13th, the maximum level registered in the Cáceres aerobiological station.

During the period studied, the number of visits to the emergency room due to asthma crisis (80 visits) increased in May, concurring with the maximum pollination period of grasses and olive trees. The higher number of visits were registered between 12^{th}-19^{th} May (25 visits). The results revealed a correlation between grass pollen counts and the number of visits due to asthma exacerbations. This correlation was significantly higher with a 3-day deviation from exposure to pollens or allergens. Positive correlation between olive pollen counts and the number of visits was also observed. This correlation was significantly higher with a day deviation from exposure to pollens, or 6-days deviation from exposure to olive allergens. These results are in agreement with previous studies analyzing the association between asthma exacerbations and grass or *Quercus spp.* pollination in the United States [23], grass pollination in Australia [24] or grass and Urticaceae pollination in Spain [25-27]. The delayed allergic response (asthma exacerbations and daily records of symptoms) after pollen or allergen exposure has been also

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observed by several authors [4, 25, 27].

The effects of air pollution (not analyzed in our study) in patients sensitized to pollen could contribute to a worse control of seasonal allergic rhinitis [28] and asthma with an increased risk of exacerbations [29,30]. Nevertheless, other studies suggest that pollination is an independent risk factor for asthma exacerbations [1, 23, 27].

Feo et al. also studied the relationship between pollen counts in Ciudad Real allergen levels and rhinoconjunctivis and/or allergic asthma in monosensitized patients to grass or olive pollen. In these studies, the authors stablished a clinical response threshold of 18 ng of total grass allergen/m^3 and 22.7 ng olive allergen/m^3 (0.9 ng Ole e 1/m^3) [2, 4]. In the present study, these grass allergen levels were registered in 5 days during May, although the threshold levels of grass pollen grains proposed by the authors (35 grains/m^3) were exceeded most of the days and in May 11th, a peak of 145 ng grass allergen/m^3 was observed.

Some authors have suggested that the pollen potency, or the quantity of allergen/grain of pollen depends on the area studied [5, 31]. In the period comprised between April 11th and May 30th, grass pollen allergenicity obtained in the present study was 40 pg of *P. pratense* allergen/pollen grain, 0.87 pg Phl p 1/pollen grain and 0.32 pg Phl p 5/pollen grain. These values are lower than the allergenic potency obtained by the HIALINE Working Group in a multicenter study developed in 10 European countries (2-2.5 pg Phl p 5/pollen grain) [31].

The olive pollen potency obtained in Cáceres during the period analyzed is higher than the obtained by other authors [5]. During 11 days, between the 5th and the 25th of May, the threshold levels of total olive allergen (maximum peak of 153 ng/m^3) and Ole e 1 (maximum peak of 19 ng/m^3) were exceeded, and the allergen values obtained per pollen grain were 159 pg olive allergen/pollen grain and 12 pg Ole e 1/pollen grain.

A positive correlation between pollen counts obtained by microscopy and allergen levels in air quantified by immunochemical methods has been found in grasses. However, in May 13th, 16th, 20th and 27th, pollen counts were high and allergen levels were low. To the contrary, in May 23th, pollen counts were low and allergen levels were high. A better positive correlation was found between olive pollen counts and the allergen levels, especially in the total allergen

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level measurement (r = 0.71). Exceptions were also found in May the 2nd (high pollen counts and low allergen levels) and May 6th (low pollen counts and high allergen levels). This low correlation has been observed in several studies, where allergen levels are maintained or even increase after the pollination period [1, 2, 6].

Pollen from trees seem to have a better correlation than pollen from grasses, being allergen levels limited to the pollination period, as occurs with olive tree [4, 5], *Platanus* [13], *Betula* [9], *Criptomeria japonica* [14], compared to grass [1, 2], *Ambrosia* [10, 11], *Parietaria* [7] and *Plantago* [12]. These differences may be due the presence of numerous grass species in this area and to a potential lack of cross-reactivity among them.

This study is focused in the spring season, although allergen measurements were made until November. There was no relevant levels of grass or olive allergens out of the pollination period. The presence of grass and olive allergens in the Cáceres environment seems to be linked to the presence of pollen. Allergen and pollen measurements could be used as indicators of asthma exacerbations.

In conclusion, this study monitors pollen grains and allergen levels during the spring time in Caceres, Spain. It reveals a significant correlation between grass and olive pollination and the increase of the number of visits to the emergency area due to asthma crisis in Cáceres. The aerobiological pattern of allergen levels in the air is comparable to the pollen counts during the grass and olive pollination period. Despite total levels and maximum peaks of olive pollen in Cáceres are lower than the grass pollen levels, the results obtained reveal a greater allergenic potency in olive pollen, a better correlation between pollen counts and allergens levels and a better association with the exacerbation of the disease in asthmatic patients.

Disclosure Statements:

The authors have nothing to disclose.

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TABLES

Table 1. Pearson correlation test between emergency department visits for asthma, pollen counts and aeroallergens levels.

	Asthma exacerbations (AE)	AE 1 day lag	AE 3 days lag	AE 4 days lag	AE 6 days lag
Grass pollen	r=0,313 (p = 0,0055)	r=0,488 ($p = 1,05 \times 10^{-6}$)	r=0,575 ($p = 4,58 \times 10^{-9}$)		
Phl p g (ng/m³)	r=0,259 (p = 0,0224)	r=0,298 (p = 0,0042)	r=0,644 ($p = 1,30 \times 10^{-11}$)		
Phl p 1 (ng/m³)	r=0,120 (p = 0,2974)	r=0,232 (p =0,0275)	r=0,525 ($p = 1,51 \times 10^{-7}$)		
Phl p 5 (ng/m³)	r=0,123 (p = 0,2856)	r=0,290 (p = 0,0054)	r=0,670 ($p = 8,60 \times 10^{-13}$)		
Olive pollen	r=0,180 (p = 0,1823)	r=0,656 (p = 0,00091)		r=0,7163 ($p = 6,20 \times 10^{-15}$)	
Ole e (ng/m³)	r=0,124 (p = 0,3609)				r=0,6078 ($p = 6,88 \times 10^{-10}$)
Ole e 1 (ng/m³)	r=0,074 (p = 0,5849)		r=0,5073 ($p = 4,54 \times 10^{-7}$)		r=0,7201 ($p = 7,97 \times 10^{-15}$)

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FIGURE LEGENDS

Figure 1A. Correlation among *P. pratense* total allergen levels (ng *Phleum* allergens/m^3) and Phl p 1 and Phl p 5 major allergens levels (ng Phl p 1-5/m^3): *P. pratense* total allergen levels *vs*. Phl p 1 r = 0.78, p < 0.0001; *P. pratense* total allergen levels *vs*. Phl p 5 r = 0.85, p < 0.0001

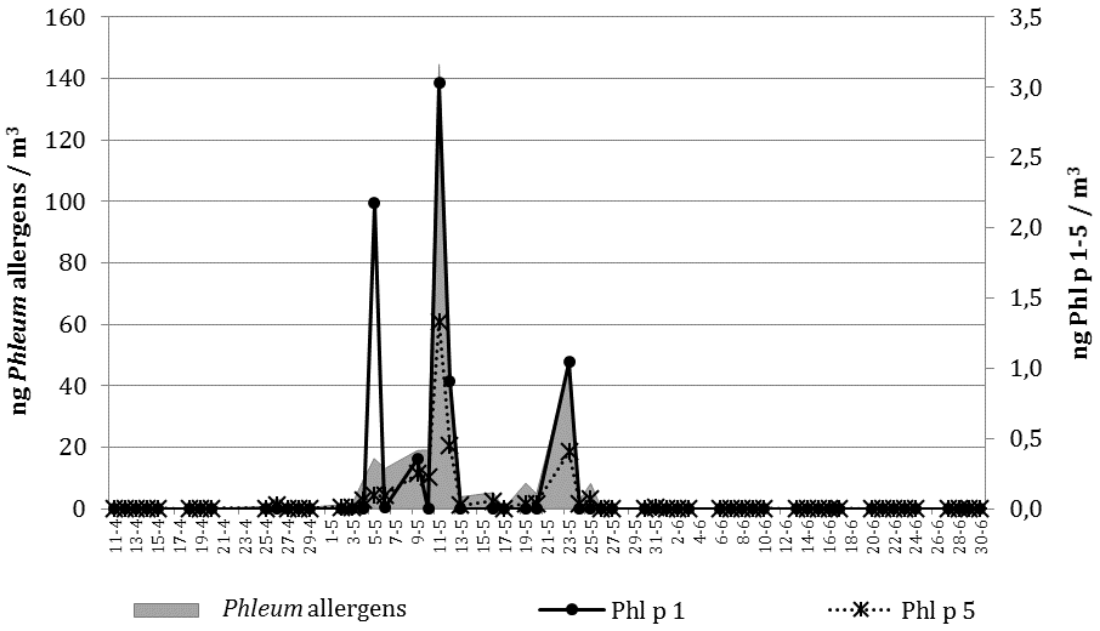

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Figure 1B. Correlation among grass pollen grain counts (Grass pollen grains/m^3) and *P. pratense* total allergen levels (ng *Phleum* allergens/m^3): Grass pollen grains *vs*. *P. pratense* allergens r = 0.57, p < 0.0001; grass pollen grains *vs*. Phl p 1 r = 0.45, p < 0.0001; grass pollen *vs*. Phl p 5 r = 0.58, p < 0.0001

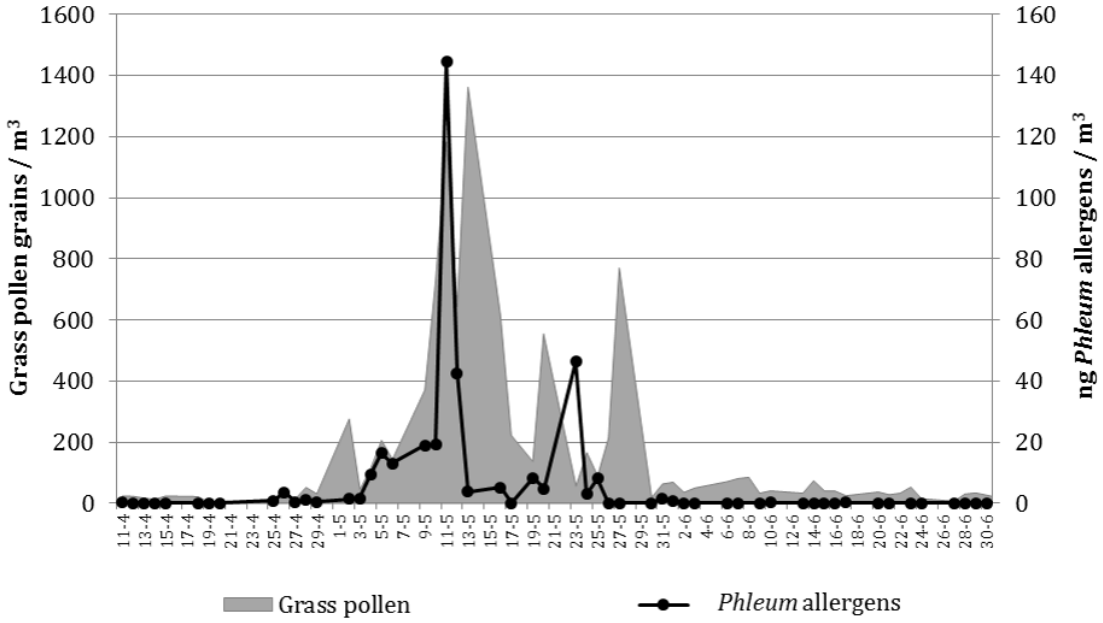

Figure 2A. Correlation among *O. europaea* total allergen levels (ng *Olea* allergens/m^3) and Ole e 1 major allergens levels (ng Ole e 1/m^3): r = 0.88, p < 0.0001

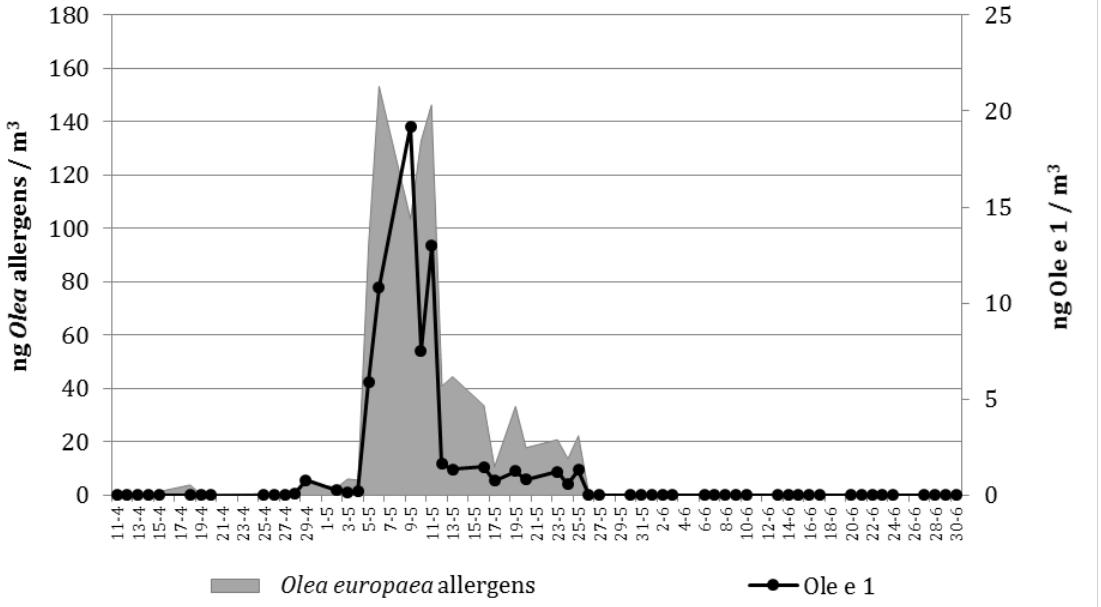

Figure 2B. Correlation between olive pollen grains counts (Olive pollen grains/m^3) and *Olea* total allergen levels (ng *Olea* allergens/m^3): r = 0.71, p < 0.0001