

Airborne Study of Grass Allergen (Lol p 1) in Different-Sized Particles

C. De Linares^{a, b} C. Díaz de la Guardia^a D. Nieto Lugilde^a F. Alba^a

^aDepartment of Botany, University of Granada, Granada, and ^bDepartment of Botany, Universitat Autònoma de Barcelona, Barcelona, Spain

Key Words

Allergens · Lol p 1 · Airborne pollen · Pollution · Allergy

Abstract

Background: The *Poaceae* family is considered one of the main causes of pollen allergy in industrialized countries. The aim of this study is to establish the dynamics of the *Poaceae* allergens and determine their distribution in the different-sized particles in the atmosphere. **Methods:** The air of Granada (southern Spain) was sampled during the pollination period of *Poaceae* using a cascade impactor and a Hirst-type volumetric collector simultaneously. The sampled airborne allergens were analyzed by indirect ELISA and field emission scanning electron microscopy. Airborne pollen was evaluated with the Spanish Aerobiological Network methodology. **Results:** *Poaceae* pollen and allergenic activity have parallel dynamics during the period of maximum pollination, which is reflected in the positive correlations between the 2 variables. In addition, the highest Lol p 1 concentrations were recorded in particle sizes lower than 3.3 μm (stage 4-F). The Spearman correlation test showed that airborne allergens are not dependent on meteorological factors, such as humidity, wind direction or sunshine, however, Lol p 1 allergen correlated positively with *Poaceae* pollen. **Conclusions:** The results of the present study confirm that the Lol p 1 allergen is detected more frequently with pollutants than with coarse particles with similar dynamics and a positive correlation

between airborne pollen and aeroallergens. Moreover, Lol p 1 is released in stable weather conditions without large changes in humidity or temperature.

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Introduction

Assessing air quality is currently one of the most promoted research lines in environmental science, especially in relation to anthropic pollutants (nitrogen dioxide, sulphur dioxide, carbon monoxide, particles in suspension and ozone), as these are the main causes of asthma in industrialized countries [1]. However, although it has been established that a quarter of the solid particles in the atmosphere are of biological origin [2, 3], few works have related air quality to pollen grains, spores, viruses, bacteria or even allergens.

Aerobiological and immunological studies indicate that spores and pollen from anemophilous plants are important biological air pollutants and that their concentration in the air is steadily increasing [1, 4].

Epidemiological studies have demonstrated that atmospheric particles with the sizes PM₁₀ (10–2.5 μm) and PM_{2.5} (<2.5 μm) in urban areas are clearly correlated with the number of hospitalizations, and consequently with respiratory allergic responses [5]. It has also been demonstrated that the particles capable of penetrating

the bronchial tree (PM10 to PM2.5) are associated with a range of adverse effects in humans, such as higher morbidity and mortality rates (particularly due to respiratory illness). However, the underlying biological causes of the health effects of particles and their chemical compositions remain unclear [6].

Pollen grains measure 15–40 µm and therefore cannot penetrate the lower respiratory tract easily [7]; however, they are the most prevalent cause of asthmatic symptoms worldwide. One of the hypotheses to explain this fact is that when the pollen grain comes into contact with the nasal mucosa it releases allergens that rapidly penetrate the lungs, provoking asthmatic symptoms [8]. Several studies indicate that in addition to pollen grains, there are also allergenic particles of plant or fungal origin with particle sizes between PM10 and PM2.5 in the air, which cause allergic symptoms [7, 9, 10].

Singh and Bhalla [11] found that, in many parts of the world, *Poaceae* pollen is responsible for pollen allergies in more individuals than any other flowering plant family. At least 40% of type 1 allergic patients are sensitive to grass pollen allergens. Several genera that belong to the subfamily *Pooideae* are considered to be the main sources of pollinosis, and are potentially the most allergenic of the genera *Phleum*, *Dactylis* and *Lolium*. Up to 12 subfamilies of allergens have been identified, and the most widely studied of these belong to the species *Lolium perenne* and *Phleum pratense*. In *L. perenne*, 7 allergens have been identified to date. Lol p 1, selected for the present study, is the most allergenic within this species. It has multiple isoforms that represent variants which appear to be immunologically indistinguishable [12], and its prevalence in people that are sensitive to *Poaceae* can reach up to 95% [13]. This allergen is an acidic protein of 35 kDa with intramolecular disulphide bridges [12] and 7 highly conserved Cys residuals, it is located predominantly in the cytoplasm [14] and is a catalyzing enzyme of the cell wall [15]. In addition, it presents a cross reaction with allergens of other *Poaceae*, such as Cyn d 1 from *Cynodon*, Phl p 1 of *Phleum* and Dac g 1 of *Dactylis* [16].

Granada (southern Spain) has a particular climate due to its peculiar topography and position in the Mediterranean basin. This area has a continental Mediterranean climate with major seasonal variations in temperature and rainfall.

This study aims to document seasonal allergenic activity in the atmosphere of Granada (southern Spain) in relation to *Poaceae* pollen and to demonstrate that there is a parallel dynamic between concentrations of grass pollen and airborne protein allergens during the pollina-

tion period of these grasses. We will also examine the particle sizes in which these allergens are detected. Scanning electron microscope/energy-dispersive X-ray analysis of the exposed filters was used to identify the size, morphology and chemical composition of the allergenic particles.

Methods

Sampling of Airborne Allergen Particles

The air content was sampled during the pollination period of *Poaceae* in spring, from 20 April to 30 June 2007, using a Cascade Impactor collector (Andersen Inc., Atlanta, Ga., USA) situated on the roof of the Science Faculty building (University of Granada) at 23 m high in the city center. Sampling took place between 12:00 and 17:00, as the continental climate in this province leads to higher temperatures during this time and therefore an increase in grass pollen concentrations [17, 18]. The largest particles are deposited in the first stages while the smallest particles pass through the collector until they are stopped by the finer filters. The first stage (stage 0) collects particles with an aerodynamic diameter of >9 µm; stage 1: >5.8 µm; stage 2: 5.8–4.7 µm; stage 3: 4.7–3.3 µm; stage 4: 3.3–2.1 µm; stage 5: 2.1–1.1 µm; stage 6: 1.1–0.6 µm; stage F: <0.6 µm.

After the peak day of *Poaceae* pollination (May 15), a preliminary qualitative analysis of the exposed filters was carried out using a high-resolution field emission scanning electron microscopy and environmental scanning electron microscopy/energy-dispersive X-ray detector together with an image analysis system. The airborne particles' morphology, chemical compositions and number-size distributions were studied.

The samples were also analyzed by an indirect ELISA [10]. For each filter, 4 circular replicates (diameter 0.5 cm) were taken on a radial pattern. As a control, 4 replicates of 1 filter with no impact were used. The filters were submerged in 125 µl phosphate-buffered saline (PBS, pH 7.4) in microplate wells for 20 h at room temperature. The discs were removed and the wells cleaned with PBS-TW (0.3% Tween 20). After blocking during 1 h at 37°C with 200 µl/well of PBS containing 1% gelatin from porcine skin (Fluka®, Buchs, Germany) and 0.3% Tween 20, the *Poaceae* antigens were detected by adding 125 µl of rabbit antibodies (Lol p 1 rabbit; Bial-Aristegui SA, Bilbao, Spain). Natural allergen from *L. perenne* pollen extract purified by standard chromatography [19], purity >98%, binds specific human IgE in ELISA and Western blotting diluted to 1:1,000 in PBS at 37°C. After 3 washes with 200 µl PBS-TW (0.3% Tween 20), 125 µl horseradish peroxidase (Polyclonal Swine Anti-Rabbit Immunoglobulins; Dako Cytomation, Glostrup, Denmark) diluted in PBS at a concentration of 1:1,000, was added and incubated in the same conditions. Further washes were carried out by incubating at room temperature and in darkness with 125 µl O-phenylenediamine tablets (OPD; Sigma, St. Louis, Mo., USA) diluted in citrate buffer (1 tablet of OPD + 12.5 ml buffer citrate + 12.5 ml distilled water + 20 µl H₂O₂). This reaction was stopped by adding 50 µl of HCl 3N. The results in all cases are expressed in nanograms of allergen per cubic metre.

A standard curve was generated of serially diluted Lol p 1 (Bial-Aristegui SA) distributed regularly in the microplate col-

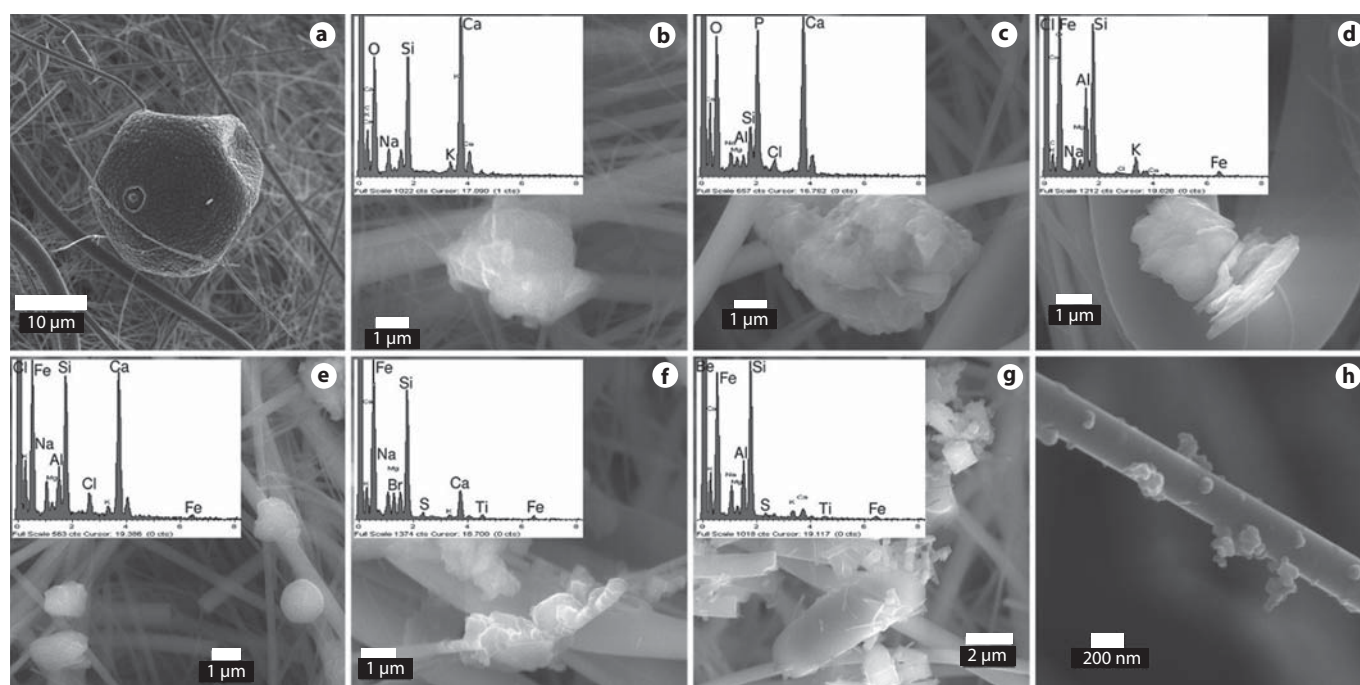


Fig. 1. Scanning electron microphotographs of the air filters of the stages and environmental scanning electron microscopy/energy-dispersive X-ray detector spectra of mineral particles collected. **a** >9 μm (stage 0); **b** >5.8 μm (stage 1); **c** 5.8–4.7 μm (stage 2); **d** 4.7–3.3 μm (stage 3); **e** 3.3–2.1 μm (stage 4); **f** 2.1–1.1 μm (stage 5); **g** 1.1–0.6 μm (stage 6); **h** <0.6 μm (stage F).

umns. We then repeated the protocol outlined above. The curve was adjusted using the mathematical software table Curve 2D V. 5.1.

Sampling of Airborne Pollen

The aerobiological samples were taken with a continuously operating volumetric collector, the Burkard spore trap [20], at the same time as the airborne antigenic particle samples. Counting was carried out with the method recommended by the Spanish Aerobiological Network [21]. Daily pollen data are expressed in grains of pollen per cubic meter. The Cascade Impactor and volumetric collectors functioned adjacently and at the same time to ensure reliable comparison of the results from the 2 samplers.

Meteorological Data

The meteorological variables included: the maximum, minimum and mean temperature, in addition to total rainfall, relative humidity, sunshine and wind direction. These data were obtained from the RAIF Meteorological Network (Red de Alerta e Información Fitosanitaria, Andalucía, Spain).

Statistical Analysis

The data were subject to a Spearman nonparametric correlation analysis to determine the degree of association between the allergenic activities recorded in the different stages of the cascade impactor, the *Poaceae* airborne pollen concentrations and the meteorological variables. The level of significance was assessed between $p < 0.01$ and $p < 0.05$.

Results

Qualitative Analysis of Particles in Different-Sized Particles

A preliminary study was carried out of the different-sized solid particles in suspension that were deposited in the cascade impactor using electron microscopy, X-ray powder diffraction, energy dispersive X-ray powder diffraction, energy dispersive X-ray spectrometry and atomic absorption spectroscopy.

This study confirmed that the particles were visually well separated by size (fig. 1). The preliminary qualitative analysis showed that the first 2 stages (0 and 1) contained the largest particles (for example, pollen grains, sand or plant/animal fragments larger than 5.8 μm). In stages 2–6, the particles, ranging from 5.8 to 6.0 μm in size, consisted of silt with chemical elements, such as Al, Mg, Ca and Fe or even high Si concentration despite its main origin is the fiberglass filters used. Finally, in stage F, soot, amorphous carbon and fuel residues were found.

Table 1. Results for the standard curve of Lol p 1

Variables in the equation	Equation	R2	ANOVA				
			source	sum of squares	DF	mean square	F
a = 3.366E-03 b = 7.894E-03	$y = a + bx^{(0.5)}$	0.966	Regr.	0.001	1	0.001	
			Error	4.649E-03	3	1.54E-06	85.8419
			Total	0.001	4		0.002

Table 2. Data as a percentage (%) and absolutes (ng/m³) for Lol p 1 in each impact stage

Stage of impaction	Particle size μm	Allergen Lol p 1, %	Total allergen Lol p 1, ng/m ³
0	>9.0	3.57	6,436.14
1	>5.8	5.76	10,094.77
2	5.8–4.7	10.29	18,042.89
3	4.7–3.3	3.49	6,123.95
4	3.3–2.1	15.65	27,448.89
5	2.1–1.1	22.21	38,943.11
6	1.1–0.7	15.36	26,943.64
F	<0.7	23.57	41,338.80
Total sum			175,372.19

Standard Curve

Table 1 shows a best fit of a second-degree equation that reflects the coefficients as well as the r^2 regression curves and ANOVA parameters. The standard curve ranged from 0 to 40 ng/ml, with a regression coefficient equal to 0.96.

Airborne Antigens

The Lol p 1 allergens totaled 175,372.19 ng/m³ (table 2) during the period analyzed. The distribution of Lol p 1 by particle size showed that the stages with the largest particles and pollen grains usually had lower concentrations of allergens than did the stages with particle sizes of less than 10 μm . In stages 0 and 1, the allergenic activity was low – only 3.57 and 5.76% of the total allergens (table 2). The highest Lol p 1 concentrations were recorded in stages 5 and F with particle sizes of lower than 2.1 μm , which can easily penetrate the lower respiratory tract.

The allergen dynamic of Lol p 1 was characterized by its continual presence over the 72 days of the analysis, although occasional increases and decreases were recorded throughout the study period (fig. 2). The highest

allergen concentration (9,118.42 ng/m³) was recorded on 30 May.

The study of the dynamics of this airborne allergen as a function of particle size (fig. 2) showed that in stages 0 and 1 there was notable allergenic activity over a relatively short period, and the highest values, 666.2 and 428.6 ng/m³, were recorded on 29 May. The highest concentrations (2,423.76 ng/m³) were in stages 4 to F, with a maximum concentration in stage 5 on 30 May.

The Spearman correlation test showed that the airborne allergens are not dependent on humidity, wind direction or sunshine. However, all stages (except stage F) and all allergens showed a negative correlation with minimum temperature (table 3). After 13 days of the 72-day study period, there was precipitation of less than 28 mm (fig. 3). For that reason, the precipitation data were not significant for the correlations.

Comparison of the Dynamics of Airborne Pollen and the Allergen Lol p 1

In 2007, airborne *Poaceae* pollen showed a marked seasonality and very high concentrations were reached in a relatively short period of time. The highest pollen concentrations were observed from the beginning of May until June. During this period, a total of 2,281 grains/m³ of pollen were collected. Both the phenological behavior and the pollen production of these grasses followed similar patterns as in previous years; the data used in this study thus fell within the standard ranges of pollen concentrations.

A comparison of the dynamics of Lol p 1 and pollen concentrations reveals that they evolved in parallel over the entire sampling period. The maximum pollen and allergen concentrations were recorded in May; the highest values were registered on 30 May (155 grains/m³ and 9,118.42 ng/m³). Based on the Spearman correlation matrix study (table 3), the Lol p 1 allergen correlated positively with *Poaceae* pollen ($r_s = 0.447$, $p < 0.01$, $n = 72$); however, the correlation was not high.

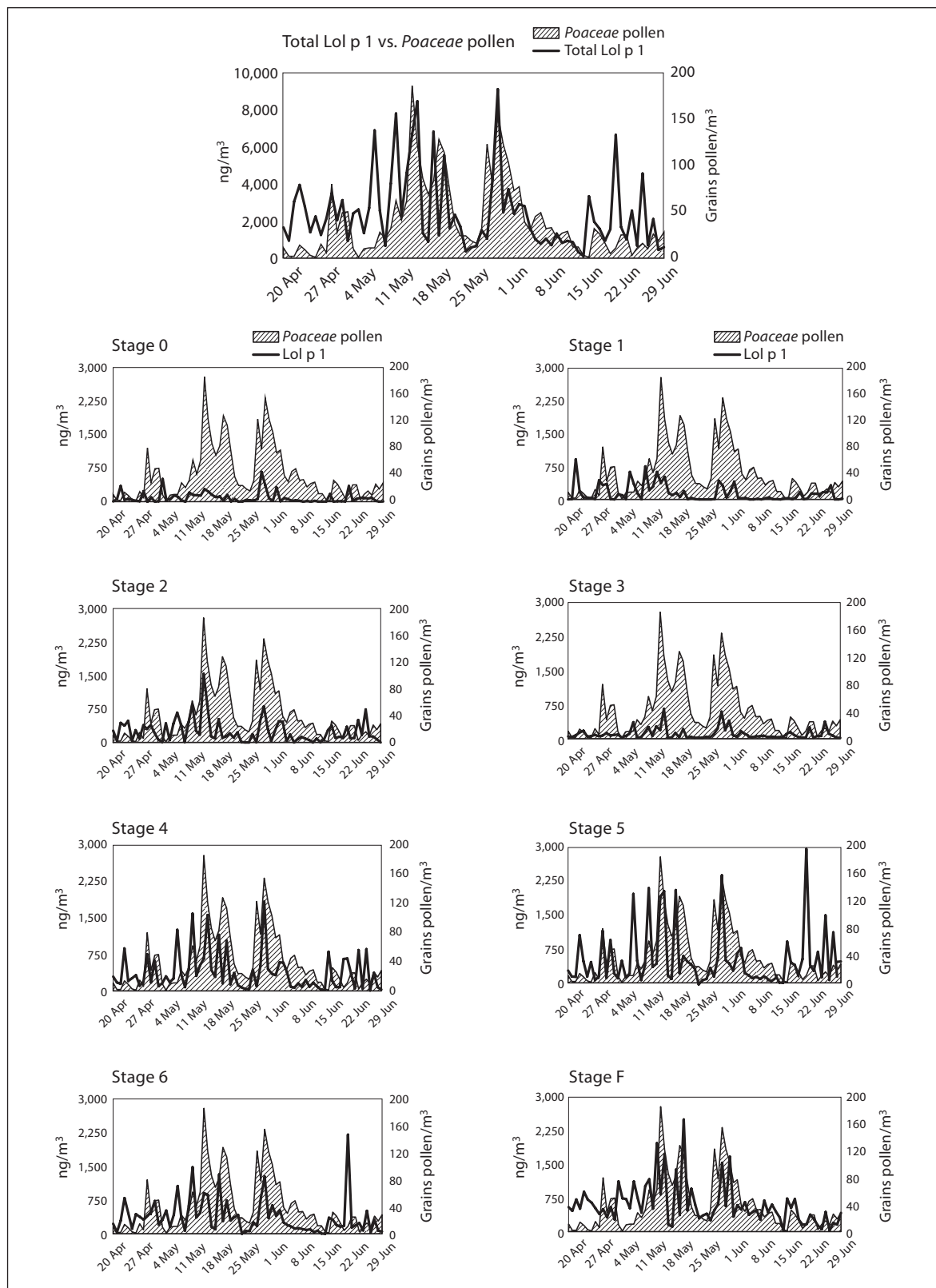


Fig. 2. Comparison of the dynamics of the total concentrations and stages of Lol p 1 (ng/m³) with *Poaceae* pollen (grains/m³) in 2007.

Fig. 3. Comparison of Lol p 1 allergen concentration (ng/m³), *Poaceae* pollen (grains/m³) and meteorological parameters (precipitation and mean temperature).

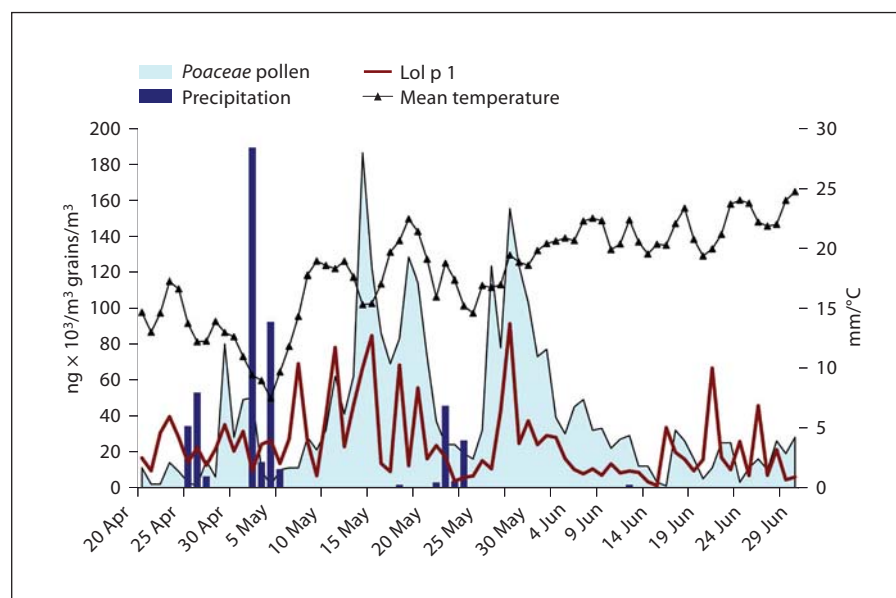


Table 3. Correlation coefficients among stages (0–F), pollen concentrations and meteorological factors

	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage F	Lol p 1	<i>Poaceae</i> pollen
Max. temperature, °C	-0.121	-0.007	0.018	-0.139	0.005	-0.020	-0.119	-0.079	-0.098	0.286
Mean temperature, °C	-0.285	-0.205	-0.142	-0.241	-0.134	-0.124	-0.276	-0.143	-0.233	0.231
Min. temperature, °C	-0.452(**)	-0.525**	-0.385**	-0.401**	-0.367*	-0.297*	-0.473**	-0.253	-0.443**	0.058
Precipitation, mm	-0.089	-0.284	-0.231	-0.211	-0.206	-0.168	-0.101	0.077	-0.139	-0.342*
Mean humidity, %	-0.223	-0.333*	-0.241	-0.242	-0.376**	-0.260	-0.181	-0.125	-0.251	-0.606**
Radiation, MJ/m ²	0.086	0.304*	0.284	0.210	0.237	0.228	0.197	0.268	0.261	0.052
Mean wind, km/h	0.009	-0.207	-0.152	-0.016	-0.112	-0.139	-0.164	-0.115	-0.139	0.002
Stage 0	1.000	0.747**	0.497**	0.662**	0.634**	0.487**	0.601**	0.473**	0.665**	0.375**
Stage 1		1.000	0.661**	0.670**	0.701**	0.577**	0.732**	0.579**	0.781**	0.312*
Stage 2			1.000	0.527**	0.770**	0.831**	0.816**	0.422**	0.809**	0.367*
Stage 3				1.000	0.731**	0.648**	0.724**	0.666**	0.790**	0.423**
Stage 4					1.000	0.890**	0.839**	0.675**	0.926**	0.511**
Stage 5						1.000	0.897**	0.677**	0.918**	0.552**
Stage 6							1.000	0.699**	0.951**	0.476**
Stage F								1.000	0.799**	0.197
Lol p 1									1.000	0.447**

** $p < 0.01$; * $p < 0.05$; $n = 72$.

The relationship between *Poaceae* pollen and allergens indicates that in the first stages (0–2) the maximum values of Lol p 1 coincided with the peak days of pollen concentration. The dynamics were also significant in stages 4 to F (PM10 and PM2.5), since both curves had similar dynamics (fig. 2). However, in stage 3 there was

hardly any allergenic activity. The Spearman correlation matrix study (table 3) showed that the Lol p 1 allergen correlated positively with *Poaceae* pollen at different stages.

Discussion

This study examines seasonal allergenic activity in the atmosphere in the urban environment. Some authors have used a gravimetric analysis of atmospheric particles to describe and control the toxicity of atmospheric contamination [22–26], since this could be associated with its composition or with the proportion of the fine fraction.

The preliminary chemical characterization of PM_{2.5}, PM₁₀ and the coarse particle presented is not sufficiently reliable to discuss. However, in this analysis we found that the coarse particles, which have a diameter of more than 5.8 µm, derived mostly from pollen, soil, plants and animal fragments. The filters obtained in stages 2–6 collected silt, such as carbonates, sulfates or iron, aluminium or magnesium oxide/hydroxide. Moreover, in stage 3, we found within the silt, phyllosilicate minerals such as illite and montmorillonite. Finally, we obtained ultrafine particles (stage F) predominantly derived from fossil fuel combustion and silt. Combustion-related particles have a core of elemental carbon coated with a layer of chemicals, including organic hydrocarbons, metals, nitrates and sulfates.

The sizing efficiency of the cascade impactor was also confirmed: pollen was only found in the first 2 stages; in the other stages, the particle sizes corresponded to the values established by the manufacturer. Therefore, as the sampling apparatus faithfully reproduced the atmospheric dust collection characteristics of the human respiratory tract, it was determined that, due to their aerodynamic size, the airborne allergenic particles can easily penetrate the lower respiratory zone and provoke asthmatic symptoms.

The results indicate that the distribution of the allergen Lol p 1 is mainly located in sizes smaller than 3.3 µm. Several authors indicated that the pollutants interact with allergens [27–29]. Our results show that allergenic and pollution-derived particles are collected in the same stage of the impactor. However, we were unable to confirm whether this is due to allergen/pollutant interaction.

The lowest activity was observed in stages 0, 1 and 3. In the first 2 cases, this may be due to the fact that the immunoassays were carried out directly on the pollen grain. Given that Lol p 1 is a cytoplasmatic protein located inside the pollen grains, if these have not yet germinated or released proteins into the microplate wells, the primary antibody in the immunoanalysis will not detect the existence of allergens. By using an adapted extraction buffer in the first incubation, the release of all allergens would be allowed. However, since the aim of this study is

to reproduce conditions in human respiratory tract, we used PBS, which does not facilitate the protein release and only the allergens easily liberated are detected. Hence, results suggest there are more free airborne subpollen particles in the atmosphere (stages 2 to F) than fast release allergens in pollen (stage 0 and 1). In this sense, a fraction of allergens in first stages would remain in the pollen grains not being detected in the analyses.

During the qualitative analysis in stage 3 silt was found; within the silt, phyllosilicate minerals were detected. The structures of all phyllosilicates are based on two-dimensional layers of polyhedra networks. Organic or water molecules can intercalate between these layers [30]. The maximum concentration of Lol p 1 in this stage was 639 ng/m³. We propose that the lower allergen concentration may be due to the fact the particles are associated with this interlayer and are not released onto the microplates during the analysis. Nevertheless, this study needs to be broadened. Interaction between allergens and phyllosilicates could be other factors explaining the high incidence of asthma in the atopic population.

Poaceae Airborne Allergens

The indirect-ELISA technique's high sensitivity is appropriate for sign amplification due to the union of 2 or more secondary antibodies for each primary antibody. The data obtained are only semiquantitative and therefore the airborne allergen and airborne pollen cannot be compared. Despite this, we have studied the dynamics of *Poaceae* allergens every spring since 2005 [31]. In 2007, airborne Lol p 1 allergens showed a similar dynamic to that described in previous years. However, as suggested by Spieksma et al. [9, 32], Schäppi et al. [33], Spieksma and Nikkels [34] and Cabrera et al. [35], our results confirm that pollen and allergenic activity have parallel dynamics during the period of maximum pollination, as reflected in the positive correlations for the 2 variables (table 3). The high values of airborne allergen obtained are similar to those obtained by the other authors with peak values reaching 13,000 ng [12, 31, 36].

The results of this study also confirm that a large part of the allergenic activity of airborne *Poaceae* pollen is localized more frequently to industrial pollutants with a particle size of less than 3.3 µm (table 2).

In the last decades, some studies have suggested that changes in the weather conditions could lead to fragmentation of pollen grains, which would then release allergens into the atmosphere. The rupture of pollen grains due to an osmotic shock during thunderstorms or under especially wet conditions has been studied. Suphioglu et

al. [37] and Davidson et al. [38] confirmed that after strong storms, there were notable asthmatic epidemics due to the osmotic rupture of the *Poaceae* pollen grain, which releases starch granules ranging in size from 2.5 to 0.6 μm into the atmosphere. In 2002, Taylor et al. [39] observed in the laboratory that grass anthers under various meteorological conditions are a site of pollen rupture and a source of fine particulate aerosols that contain pollen allergens.

Our study thus demonstrates that pollen grain allergens are released into the atmosphere in regions with low rainfall. During the study period, the weather was stable and there were no sudden temperature or humidity changes. In our study region, the outbreaks of asthma associated with storms or with sudden humidity and temperature changes are not common; therefore, pollen allergens must also be released in stable weather conditions.

The presence of pollen allergens in association with orbicules was recently verified in certain species using immunocytochemical methods [40–43]. The existence of these orbicules in pollen exines of several grasses has been documented [43–45]. According to some studies, these orbicules are released and become part of the airborne submicronic fraction [46]. Evidence also suggests that some allergens may become airborne as aerosol from sap, for example when lawns are cut [47]. These factors may explain why the highest allergen concentrations in the samples contained particles of less than 3.3 μm . The correlations obtained from our results suggest that allergens are released during the main pollen season, although lower correlation values indicate that the behavior of these allergens in the atmosphere is different than the dynamics of the pollen grains.

Spieksma et al. [32] attributed this phenomenon to the collection methods, which may induce high concentrations if the pollen ruptures during collection. However, our analysis is consistent with the findings of Solomon et

al. [48] and De Linares et al. [10], who found no fractured pollen in the filters, based on an MEB analysis.

According to our results, pollen and allergenic activity show parallel dynamics during the period of maximum pollination; this was also observed by De Linares et al. [10] as regards *Olea europaea* pollen. However, *Lolium* and *Olea* allergens behave very differently in their airborne profiles. In the study of the dynamics of *Olea*, important differences appeared between the concentrations of allergens and pollen during the pre- and postseasonal periods; in the study of *Lolium*, this was not the case. Several studies have demonstrated that Ole e 1 has a certain homology with allergens of the family *Oleaceae* and other taxonomically unrelated genera such as *Plantago* (Pla a 1), *Lolium* (Lol p 11) and *Chenopodium* (Che a 1) [49]. However, group 1 of *Lolium* did not cross-react with weeds (*Plantago*, *Rumex*, *Helianthus* and *Parietaria*), trees (olives) or members of the *Panicoidea* subfamily [50]. This effect may be observed in the study of airborne *Poaceae* and *Olea* allergens. Whereas the dynamic of Lol p 1 and *Poaceae* pollen was broadly similar throughout the spring, De Linares et al. [10] demonstrated a similar dynamic only during the main pollen season.

In conclusion, this study reveals that there is a similar dynamic and a positive correlation between the allergenic activity of Lol p 1 and *Poaceae* pollen; the allergens are released in stable weather conditions, that is, without significant humidity or temperature changes; an analysis of the different-sized solid particles in suspension showed that the allergens are detected more frequently with pollutants than with coarse particles.

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