

Trisetum paniceum (Wild Oats) Pollen Counts and Aeroallergens in the Ambient Air of Madrid, Spain

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Key Words

Grass allergens · Aeroallergens · Allergenic activity · Pollinosis · *Trisetum paniceum* · Aerobiology

Abstract

Background: Madrid has a short but intensive grass pollen season, in which 79% of the total grass pollen load is released from the middle of May to the middle of June. The main objectives of this study were to quantify *Trisetum paniceum* (wild oats) aeroallergen in the atmosphere in Madrid from February to December 1996 and to correlate the aeroallergen concentrations with grass pollen counts. **Methods:** Two different samplers were used to assess allergen exposure; a Burkard spore trap was used to collect pollen grains and a high-volume air sampler to collect airborne particles. A total of 182 air filters were collected and extracted in 1 ml of PBS and analysed by ELISA inhibition, using pooled sera from highly allergic individuals. **Results:** *T. paniceum* aeroallergens were detected not only during the grass pollen season, but also before and after. Wild oat allergens had two main peaks of 1 and 1.9 $\mu\text{g}/\text{m}^3$, occurring in late May and July, respectively. The time series analysis estab-

lished the existence of lags between the two main variables pollen counts and aeroallergen activity. Analysis of the data by the Spearman rank test and linear regression showed a weak correlation between grass allergenic activity and grass pollen counts (Spearman's $\rho = 0.29$). Data obtained from time series analysis demonstrated that grass allergenic activity correlated strongly with current and 5-week-old grass pollen grain counts ($r^2 = 0.73$). **Conclusions:** Wild oats allergenic activity was detected during the entire year and not only during the pollen season. This fact is an important aspect to be considered in the clinical follow-up and treatment of grass pollen-sensitised patients in Madrid.

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Introduction

It has been shown that clinical symptoms of pollen-allergic patients correlate with atmospheric pollen grain counts [1]. However, the existence of extra-seasonal pollen aeroallergen activity has been demonstrated, and pollen-sensitive patients often report clinical symptoms outside of the pollen season [2, 3]. Therefore, it has been sug-

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gested that pollen counts may not encompass the total aeroallergen exposure [4].

1996 was the first year in which there was normal rainfall after a 7-year drought in Spain. The pollen counts prior to 1996 were considerably lower due to this lack of rain. Pollen grains represent the most important cause of respiratory allergies in Madrid; 64% of patients with rhinoconjunctivitis and 52% of patients with asthma are sensitive to grass allergens [5].

In the atmosphere of Madrid, there is a predominance of small-pollen grain grasses (diameter <30 µm), as has been shown by pollen counts (mean of 63% of all grass pollens) [5]. In a previous study, Subiza et al. [6] demonstrated that *Trisetum paniceum* was the most common grass species producing small pollen grains in the Madrid area. *T. paniceum* has been described as one of the major causes of type I allergy in this region. One hundred percent of grass-allergic patients are sensitised to this grass species [6]. *T. paniceum* pollen has a high proportion of IgE-reactive proteins, including an atypical proportion of basic components which are not detected in other phylogenetically related pollens [7]. The latter study demonstrated cross-reactivity between *T. paniceum* and other grass pollens, such as *Lolium perenne*, *Festuca elatior*, *Poa pratensis*, *Dactylis glomerata*, *Phleum pratense* and *Holcus lanatus*.

Several aeroallergens have been quantitated immunochemically, including *Ambrosia* [2], birch [8], oak [3] and *Parietaria* [9]. It has also been shown that the allergenic activity can be transported by small particles. The clinical implications of plant allergens being carried by small particles have generated significant debate in recent years. This activity can be detected prior to the pollen season and may continue after pollination is completed, thus contributing significantly to the total allergen load. Since these particles are respirable, they can penetrate deeply into the airways and may cause exacerbation of asthma. However, little is known about the prevalence of these particles in the atmosphere, their geographic and longitudinal variations or their detailed chemical composition. In addition, their exact origin is unknown. However, several mechanisms which combine variables, such as atmospheric disturbances, habitat and pollution, have been proposed. Due to the fact that in Madrid, grass pollen-allergic patients experience allergic signs and symptoms outside of the pollination period, we consider that the allergen content in the atmosphere should be investigated during the entire year. Madrid has approximately 4.5 million inhabitants, and several million tourists visit the city every year. Therefore, we believe that both pollen counts

and atmospheric grass allergen content should be regularly determined and included in aeroallergen forecast reports.

The objective of this observational study was to quantify the allergen content of wild oats in the atmosphere in Madrid during 1996 and to correlate the results with grass pollen counts.

Materials and Methods

Pollen Counts

Grass pollen grains were sampled with a volumetric spore trap according to a previously described technique [10]. A Burkard trap (Burkard Manufacturing Co., Rickmansworth, Herts., UK) was used during the sampling period, which lasted from February to December 1996. The trap was installed on a 20-metre-high roof located in the eastern part of the Madrid urban area, with no parks or gardens nearby. Pollen identification was conducted by expert technicians and was based on comparison with reference slides and photographs, as described by Hyde and Adams [11].

The 1996 season was selected because of the availability of the equipment, which was not previously available, and the fact that the drought period ended in the autumn of 1995. Forecasts suggested an intense spring pollen season.

Air Sampling

The Air-Sentinel, a volumetric air sampler, was used for aeroallergen quantification (Quan-Tec-Air Inc., Rochester, Minn., USA) [12]. This device was used to collect aeroallergens in several previous studies [13–15].

This machine was run continuously from February to December 1996. It incorporates a multi-stage centrifugal blower (Kooltronics, Hopewell, N.J., USA) and a custom-built stainless steel filter head assembly (HiMec Inc., Rochester, Minn., USA). The Air-Sentinel was placed 2 m away from the Burkard spore trap on the same flat roof and operated at a flow rate of 10 m³/h. Airborne particles were collected onto polytetrafluoroethylene filters (Quan-Tec-Air) rated with a 99.9% efficiency at 0.3 µm. The overall average sampling time during the pollen season was 36 h, compared to 72 h outside the season, which represents a range of 360–720 m³ of air per sample. After the sampling period, the filter was sealed in a plastic bag and stored at 4°C. The filters are composed of stretched Teflon with a polyester support backing. A total of 182 air filters were sampled and extracted in 1 ml of PBS.

Immunochemical Quantitation of Airborne Allergens

The polytetrafluoroethylene membranes were separated from the backing and placed in a 13 × 100 mm tube along with the buffer. The support backing was discarded and the tube was stirred until the entire sample was wet. The sample was kept overnight at 4°C, mixed again the next day and centrifuged. To prevent a loss of allergenic activity, the samples were eluted and processed immediately.

The eluted samples were tested in duplicate for allergenic potency by ELISA inhibition. Pooled sera from 30 allergenic individuals selected on the basis of a history of seasonal symptoms, positive skin test to grass pollens and the presence of high titres of specific IgE to grass pollen allergens were used. The total IgE level of the serum pool used was 476 kU/l, as measured by the Pharmacia CAP system. The

specific IgE level to cross-reacting grasses was class 4. The serum pool was used at a final dilution of 1/100 (v/v). The immunochemical quantitation of these filters was conducted following methods previously described [3].

Briefly, a *T. paniceum* extract with a protein concentration of 2.78 mg/ml (Immunotek, Madrid, Spain) was diluted in sodium bicarbonate buffer (0.2 M, pH 9.2) to a final concentration of 10 µg/ml; 100 µl were coated to the wells of flat-bottomed microtitre plates (Immulon 4, Dynatech, Chantilly, Va., USA). After overnight incubation, wells were washed and blocked with 1% human serum albumin (HSA) in PBS at room temperature for 30 min.

Each filter extract was mixed with an equal amount of the serum pool to a final volume of 125 µl and shaken for 4 h at room temperature. After this inhibition period, 100-µl aliquots were incubated overnight in the previously coated plates. After washing, 100 µl of anti-IgE-horse radish peroxidase (HRP) (Ingenasa, Madrid, Spain) at a concentration of 1 µg/ml were added and left to stand at room temperature for 4 h. After washing, the peroxidase reaction was evaluated by incubation with a mixture of 0.16 M acetate buffer (pH 6.1), tetramethylbenzidine (TMB) in dimethyl sulphoxide (20 µl) and 1 M H₂O₂ (2 µl). The enzymatic reaction was stopped with the addition of 100 µl of 2 N sulphuric acid. Optical densities were finally read at 450 nm in a microplate reader (Titertek Multiskan, Finland). The percentage of IgE inhibition was calculated using a reference inhibition curve established with an internal standard of *T. paniceum*. The lower limit of detection of the assay was 5 ng/ml. The standard curve used was determined after 3 duplicate and parallel trials and included 9 points. The results of these trials were similar, with a <10% variability. A serum pool from 5 non-allergic individuals and sera from *Dermatophagoides pteronyssinus*-positive, grass-negative individuals were used as negative controls. Furthermore, we also included an unexposed filter in the inhibition studies, which showed no inhibition. The negative control sera did not bind any specific IgE to *T. paniceum* allergens in the solid phase. The working conditions were identical in all these experiments.

Cross-Reactivity Studies

Specific IgE inhibition ELISA experiments were used to assess potential cross-reactivity between *T. paniceum*, *Olea europaea*, *Che-nopodium album* and *Platanus hybrida*. Briefly, plastic microtitre plates (Immulon 4) were coated with *T. paniceum* extract (1 µg of protein/well) overnight. Several dilutions were made from the *T. paniceum*, *O. europaea*, *P. hybrida* and *C. album* extracts to generate inhibition curves. Every dilution was incubated, in duplicate, for 2 h at room temperature with a serum pool containing high titres of specific IgE to *T. paniceum*. Afterwards, the dilutions were transferred to the *T. paniceum*-coated plates and incubated overnight. After washing, 100 µl of peroxidase-labelled anti-human IgE were added and left to stand for 30 min at room temperature. After washing, the reaction was developed for 30 min and then stopped with 1 N sulphuric acid. The results were read at 450 nm in a microplate reader and stopped as previously described. The allergenic activity of *T. paniceum* extract was evaluated by establishing 50% inhibition. Maximal inhibition was calculated with three other extracts.

Statistical Analysis

The data were analysed by the Spearman rank test and linear regression to investigate the correlation between pollen and aeroallergen levels. Time series analyses were performed on the weekly average data to establish relevant lags between these two variables

using a dynamic regression model [16]. The dynamic regression model (ARIMA) evaluates how the output (aeroallergen levels) is linearly related to current and past values of the input (pollen grain levels). Days when neither pollen grain counts nor airborne allergen determinations were detected were also included in the correlations. The objective of the time series analysis was to identify significant lags to be included in the mathematical model using the simple and partial autocorrelation functions. The estimation of the ARIMA models is complicated and uses non-linear optimisation algorithms to calculate the values of the parameters with the maximum likelihood ratio. The result of the estimation is a table analogue to that of the regression. This table provides the estimated values of the parameters and their T statistics and standard errors. Other analysed variables included rain, relative humidity, temperature, wind, particulate material, O₃, SO₂, NO₂, CO and CH₄.

Results

Pollen Counts

March, April, May and June were the months in which the maximum pollen concentration was measured and accounted for 80% of the total annual pollen. The main grass pollination period ranged from 14 May to 21 June. *O. europaea* pollen grains were also identified at the beginning of this period. Grass pollen levels peaked on June 1 (552 grains/m³), which was the highest level recorded in the previous 5 years (fig. 1). The total amount of grass pollen recorded for the entire year was 6,588 grains/m³, with a relative frequency of 17.73%. Forty-four pollen types were identified in Madrid in 1996.

Cross-Reactivity Studies

Approximately 0.1 µg/ml were necessary to reach the 50% inhibition point for the *T. paniceum* extract. However, the 3 other extracts did not reach 50% inhibition. The highest percentage of inhibition reached by *O. europaea*, *P. hybrida* and *C. album* were 18, 21 and 28%, respectively (fig. 2). These inhibition levels were reached, in all cases, at 6.24 µg/ml. These data exclude a potential contribution of *O. europaea*, *P. hybrida* and *C. album* pollen to the *T. paniceum* aeroallergen levels detected in the study.

Immunochemical Quantitation of Airborne Allergens

Variable amounts of airborne *T. paniceum* aeroallergen were detected during the analysed season. In addition to detecting *T. paniceum* aeroallergens during the pollen season, allergenic activity was also detected before and after the pollination period (fig. 1). *T. paniceum* aeroallergens showed two main peaks (1 and 1.9 µg/m³), one in late May and the other in July.

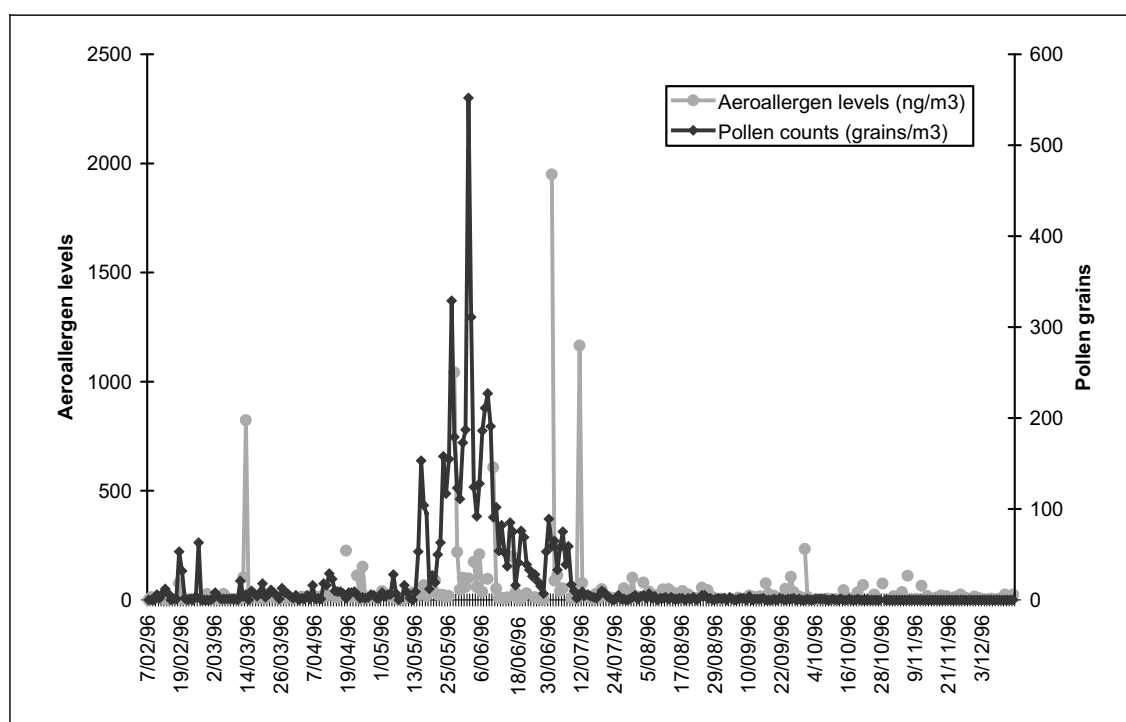


Fig. 1. Allergenic activity and grass pollen grain counts during 1996 in Madrid, Spain.

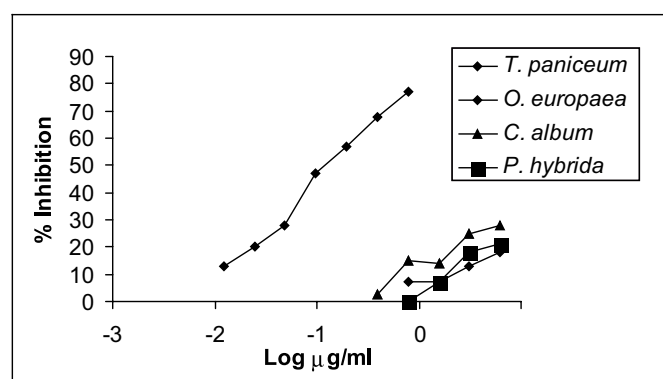


Fig. 2. ELISA inhibition assays using *T. paniceum* in the solid phase. *T. paniceum*, *O. europaea*, *P. hybrida* and *C. album* were used as inhibitors in the fluid phase.

Statistical Analysis

Analysis of the data by the Spearman rank test and linear regression showed a correlation between grass allergenic activity and grass pollen grains ($r_s = 0.29$). Data obtained from time series analysis demonstrated that grass allergenic activity correlated with current and 5-week-old grass pollen grain counts ($r^2 = 0.73$). The initial exploratory bivariate analysis showed a highly significant correlation between the outcome variable allergens and the pollen grain levels over the previous 5 weeks. The final

multivariate analysis showed that only current pollen grain levels and those of the previous 5 weeks were significantly correlated with the outcome variable allergens. The other explanatory variables (atmospheric and pollutants) did not enter into the definitive regression model, except for temperature ($p = 0.004$) and humidity ($p = 0.020$). The correlations between allergen levels and other atmospheric variables and pollutants were not included, because they did not reach statistical significance.

Table 1. Other potential sources of allergens beside specific pollen grains

Allergen source	Reference
Cross-reactivity between taxonomically related and non-related pollens	7
Contribution of leaves or stems	3
Starch granules from ruptured pollen grains	18
Orbicules from the dehiscing anthers	19
Allergens that are carried on the pollen grains themselves	20
Allergens transported by naturally formed environmental particles (microdrops of water)	20
Allergens carried by pollutant particles	21, 22

Discussion

Due to the copious rainfall during the previous fall and winter seasons, 1996 was characterised by high levels of atmospheric pollen concentrations. Grass pollens appeared in the atmosphere, rose sharply to extremely high levels and returned to lower counts after 5 weeks.

We describe the presence of *T. paniceum* aeroallergens before, during and after the pollen season, which is in accordance with other studies which have shown similar results for other allergens [2, 8, 17]. As has been indicated in several studies, the existence of grass pollen allergens outside of the pollination period indicates that there are other sources of allergens besides the pollen grains which can carry allergenic activity. Several possibilities have been suggested as an explanation for these findings (table 1). Meteorological factors may play an important role in the atmospheric presence of grass pollen allergens associated with non-pollen particles [23]. The transfer of allergens from pollen grains to the environment may provide an explanation for the presence of grass pollen aeroallergens outside of the pollen season. Grass pollen allergen-carrying particles may become re-suspended in the air by meteorological events such as changes in wind directions and thunderstorms.

A general correlation between elevated pollen counts and allergen activity has been demonstrated for *Ambrosia* [24]. In the present study, we detected grass allergen levels when grass pollen grains were visible in the collector, but there were occasions when allergen levels were high in the absence of pollen, as other authors have described for *Ambrosia* [2, 4] (table 1). These investigators described that aeroallergen activity correlated better with symptom

scores than did pollen grain counts. They also found that *Ambrosia* allergenic activity peaked later than the respective pollen grain counts. These observations could explain the poor correlation between pollen grain counts and *Ambrosia*-induced asthma symptoms, which are often observed after the pollination period [2, 4]. Solomon et al. [4] demonstrated by skin test and in vitro tests that the *Ambrosia* allergen content in micronic or sub-micronic particles is clinically relevant.

Our results suggest that after an intense pollination day, allergens are released from the pollen grain and re-suspended some time later. Ricca et al. [25] have shown a significant inflammatory reaction in patients throughout the pollen season, even on the days with low pollen counts. This fact could be explained by the presence of allergenic activity outside of the pollination period. A constant exposure to allergens, even when they do not produce symptoms, seems to promote and maintain the inflammatory response. According to these authors, this inflammatory response is associated with the expression of ICAM-1 in the epithelial cells or in the inflammatory infiltrate in the mucosa. Bachert et al. [26] evaluated the release of cytokines under natural conditions and found that allergic rhinitis represents a persistent inflammation with eosinophil activation and up-regulation of IL-1 β during the pollen season and thereafter. In the present study, the grass allergenic activity detected during the whole year could explain the persistence of high titres of specific high IgE in the serum of grass pollen-sensitised patients and baseline inflammation. Therefore, it is important to consider the length and intensity of the grass season in the clinical follow-up and treatment of patients, as has been proposed by Ricca et al. [25].

Subiza et al. [6] observed a lag of 7–14 days from the appearance of the first pollen grains in the air in Madrid until the first symptoms were registered on daily symptom cards. This time lag was even longer for the appearance of asthma symptoms [6]. The results of the present study could explain the time lag between pollen counts and symptoms in grass-sensitive patients [6, 27].

In Madrid, the grass pollen season can start as early as February. In this month, we were able to identify some grass pollen grains, most likely of *Poa annua* [5]. Another source of antigenic activity outside the pollen season could be grass juice derived from leaves and stems, as reported in previous studies [28, 29].

We believe that grass aeroallergen levels ranging from 1 to 2 $\mu\text{g}/\text{m}^3$ are sufficient to sensitise and cause symptoms in genetically prone individuals residing in Madrid. This assumption is based on the fact that similar allergen

levels are capable of inducing symptoms [24] and because grass allergy is the most important cause of allergic respiratory symptoms in this area [5]. These levels are similar to those measured in barns or other occupational settings [13].

An important finding of our study is that grass aeroallergen activity can be explained by current and 5-week-old grass pollen counts. Attention is drawn to the fact that there were three allergen peaks above 500 ng/m³ outside of the pollination period, 5 weeks after the grass pollen peaks. In September, after the pollination period, we detected some grass allergenic activity, as well as *C. album* pollens, which were visible in the collector. We performed a cross-reactivity study between *T. paniceum* and *C. album*, *O. europaea* and *P. hybrida* and confirmed that none of the latter three pollen extracts reached 50% inhibition, while *T. paniceum* reached 80% at a concentration which was approximately 10 times lower. This fact reinforces the idea that the quantified allergens in the atmosphere of Madrid are grass derived, since high levels of *O. europaea*, *P. hybrida* and *C. album* would be needed to produce a significant inhibition. This is also suggested by the test for parallelism, which indicates important differences in the allergenic composition of the extracts (fig. 2).

A significant feature of our study was the 5-week lag between grass pollen counts and grass allergenic activity observed throughout the year. This indicates a consistent relationship between these variables. This phenomenon occurred consistently, regardless of meteorological or polluting influences. We evaluated some of these influences (rain, relative humidity, temperature, wind, particulate material, O₃, SO₂, NO₂, CO, CH₄) and found no significant correlation between the variables except for temperature ($p = 0.004$) and relative humidity ($p = 0.02$). We did not investigate the exact reason for these significant correlations, but we suggest that an increase in temperature and humidity could facilitate the release of allergens from the pollen grain and their dispersion into the ambient air. Other potential factors could be the natural degradation process of the pollen grain, agricultural activities related to wheat or other cereals or desiccation of the grass plants, with the additional release of small allergenic particles into the air.

The immunochemical method of assessing allergen exposure takes into account the contribution of cross-reactive allergens and other allergen sources. The assessment of airborne allergens would provide more direct and realistic data about aeroallergen exposure of sensitised and non-sensitised individuals. However, performance of these immunochemical techniques to quantitate airborne

allergens is not conducted on a routine basis. There is a clear need for more basic research in sampling small particles from the atmosphere and in immunochemical techniques for quantifying aeroallergens, particularly for the development of quick and reliable methods that could be routinely applied to supplement information from pollen counts. Until these are available, pollen counting will still play an important role for allergologists in predicting possible clinical manifestations. However, there is now clear evidence that a significant quantity of aeroallergens can be detected outside of the pollen season and that allergenic activity is carried in small, non-pollen plant parts.

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Note Added in Proof

Recently data support the presence of an out of season stimulus in pollen-sensitised patients (Smurthwaite L, Walker SN, Wilson DR, Birch DS, Merrett TG, Durham SR, Gould HJ: Persistent IgE synthesis in the nasal mucosa of hay fever patients. Eur J Immunol 2001; 31:3422–3431).

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