aerobiology

MDPI

Article

Seasonal Pollinosis Due to Kans Grass Pollen: Prevalence and Immune-Biochemical Approach

Tanmoy Basak [1,*] and Kashinath Bhattacharya [2]

[1] Department of Botany, Midnapore College (Autonomous), Midnapore 721101, West Bengal, India
[2] Environmental Biology Laboratory, Department of Botany, Visva-Bharati, Santiniketan, Bolpur 731235, West Bengal, India; kashinathb23@rediffmail.com
* Correspondence: tanmoy.basak1@gmail.com; Tel.: +91-6291884823

Abstract: Background: It has been recognized from the early days of allergology that aerobiological investigations have an important role to play in the development of respiratory allergic diseases. An increasing number of allergic complaints occurred among the atopic population during the blooming season of Kans grass, *Saccharum spontaneum* (SS), an obnoxious weed growing in and around suburban West Bengal. The present study aimed to identify SS pollen as a potential aero allergen through aerobiological, clinical, statistical, and biochemical analyses. Methods: An aerobiological survey was conducted for 2 years followed by a clinical diagnosis of 134 local atopic patients suffering from a respiratory allergy by a standard questionnaire survey and the skin prick test (SPT) using SS antigens. The antigenic protein profile was analyzed by SDS-PAGE and the allergizing potential of this pollen was investigated by an in vitro enzyme-linked immunosorbent assay to recognize the presence of the sero-reactive proteins which were the suspected cause of the respiratory allergy. A Box-plot and regression analysis were performed to establish the significance of clinical data. Results: SS pollen was found to evoke about 70.14% sensitivity among the atopic population causing early spring hay fever, allergic rhinitis, and seasonal allergic conjunctivitis. A regression analysis for the pollen antigen for estimating the total IgE value of a patient's sera from their specific IgE value was a novel approach by our study. The antigenic extract of pollen resolved into more than 15 distinct protein bands ranging from 14.4 to 116 kDa, some of which were found to be glycosylated. The results showed that SS pollen has a significant presence in the atmosphere, which may trigger an allergic response in immunocompromised patients. Conclusions: This is, to our knowledge, the first attempt to identify allergens from Kans pollen causing seasonal pollinosis among the Indian atopic population using an immuno-clinical approach.

Keywords: aeroallergen; aerobiological investigation; grass pollen allergy; seasonal pollinosis; *Saccharum spontaneum*; regression analysis

Citation: Basak, T.; Bhattacharya, K. Seasonal Pollinosis Due to Kans Grass Pollen: Prevalence and Immune-Biochemical Approach. *Aerobiology* **2023**, *1*, 37–53. https://doi.org/10.3390/aerobiology1010004

Academic Editor: Athanasios Damialis

Received: 15 April 2023
Revised: 7 September 2023
Accepted: 13 September 2023
Published: 20 September 2023

1. Introduction

It has been well established that grass pollen is a major bio pollutant that causes allergic disorders in susceptible individuals in many parts of the world. Grasses (family Poaceae) are ubiquitous plants throughout the world and cover almost 20% of global ground vegetation representing more than 11,000 recognized species with a wide distribution [1–6]. Pollen of grasses evokes allergic responses in 20% of the general population and 40% of atopic individuals [7–12]. Grass pollen contributes a significant part of the bioaerosol as they are mostly anemophilous (wind-pollinated), except for some cleistogamous species and a smaller number of entomophilous species [13]. Furthermore, this pollen is small in size (average diameter: 20–40 µm), and versatile anther helps in easy dissemination of large quantities during a short period pollen [14,15]. The aerial concentration and species diversity of airborne grass pollen differ according to the diurnal patterns, seasonal and meteorological conditions, and geographical location [16–18].

Grass pollen allergens are grouped according to their molecular structure and biological function [18–20]. They are named according to the official system of nomenclature (www.allergen.org, accessed on 23 January 2023), viz. Phl p1 is grouped under grass group allergen 1 from *Phleum pratense* (commonly known as timothy grass) [6]. Grass allergen group 1 and 5 allergens are identified as dominant allergens, considering their abundance and allergic potency [21,22] Attempts have been made to study grass pollen allergens starting around the 1960s, and since then, more than 89 allergens have been reported [5,10,23]. Recent advancements in molecular biology techniques have allowed extensive characterization of allergenic molecules as well as the synthesis of recombinant allergens [11,19,24]. The hypersensitive immune response to several grasses' pollen has been studied extensively over more than three decades in India, as well as around the World [18,25–30]. In India, the earlier investigations and diagnoses were restricted to aerobiological surveys [31–33] and screening the patients by skin tests with pollen extracts of grasses, respectively, except for a few immunoclinical studies as reported by Kumar et al. (1998), Varma et al. (2000), Singh & Shahi (2008), and Bhattacharya et al. 2018 on grass pollen allergens [22,34–36].

The Poaceae (grasses) family comprises the highest number of flowering plant species shading a huge number of pollen grains in the atmosphere, which are easily carried out by wind during the pre-monsoon and post-monsoon seasons [18,28,33,37,38]. Prediction of the grass pollen (aeroallergen) concentration in the atmosphere and precise identification of the causative allergic species is necessary for acute allergy sufferers when the patient is treated with allergen-specific immunotherapy [39]. Moreover, grass pollen is the major cause of pollinosis in Europe [9,40–43], and one of the most significant airborne allergen sources globally [15,18,40]. Earlier investigations on the predominance of grass pollen in the atmosphere, identification of their allergic potency, and molecular characterization of individual proteins were usually restricted in Europe and the USA for the last few decades [5,10,11,14,15,20,22,44–49]. However, only a few attempts were made to evaluate the allergenic potential of predominant grass pollen in tropical countries such as India. Grass pollen allergens from five species, namely, *Imperata cylindrica* (cogon grass), *Cenchrus ciliaris* (bunch grass), *Pennisetum typhoides* (pearl millet), *Sorghum vulgare* (sorghum), and *Cynodon dactylon* (Bermuda grass) have been identified and comprehensively characterized [22,36,50–54]. To lower the incidence of societal expenses and enhance quality of life, attention must be devoted to one of the most crucial health issues of the twenty-first century: people's susceptibility to allergens.

Saccharum spontaneum L. (Kans grass in Bengali) is a perennial, polymorphic grass species believed to have originated from native to the Indian subcontinent. SS has the potential to become a serious invader of cultivated land, often resulting in its abandonment. SS is a common weed in West Bengal, India growing profusely during the autumn season, i.e., from August to November (Supplementary Figure S1) along riverbanks, roadsides, and railroads, on waste ground, and along the banks of lakes and ponds [55], while blooming from September to mid-October, releasing large quantities of pollen into the atmosphere. Due to the stenopalynous palyno-morphological characteristic of Poaceae pollen, it is difficult to distinguish between pollen grains of the different genera and species of grasses with the help of light microscopy alone [39]. It is, thus, impossible to estimate the individual load of grass pollen contributions of the different species to the pollen spectrum in the atmosphere. Moreover, there are more than 11,000 grasses found in India, thus making it challenging to characterize each grass's pollen allergens [35].

Studies conducted earlier have proved the allergizing potentiality of *Saccharum spontaneum* pollen based on aerobiological surveys and SPT on respiratory allergy patients [28,56–58].

2. Materials and Methods

2.1. Aerobiological Monitoring

Biomonitoring of airborne pollen flora was performed with the help of "Burkard portable volumetric sampler" (Burkard Manufacturing Co., Ltd., Hertfordshire, UK) in

Santiniketan. The sampling was carried out for two consecutive years from January 2013–December 2014. The sampler was placed somewhere about 2–3 m above ground level which is measured to be the inhalation atmosphere of humans.

2.2. Sampling Methods

Burkard personal air sampler (air suction rate = 10 L/min) was operated for 10 min for monitoring and assessment of airborne pollen spectra at the experimental sites. In Santiniketan, the sampler was simultaneously operated for two consecutive years (January 2013 to December 2014) at different places (Figure 1). Air sampling was conducted at three different time intervals: morning (09:30 h–10:30 h), afternoon (12:30 h–13:30 h), and evening (19:30 h–20:30 h) at weekly intervals [59]

Figure 1. Aerobiological sampling by Burkard personal volumetric sampler at different sampling locations of Santiniketan.

A microslide of 1 cm (RivieraTM −1″ × 3″) thickness smeared with glycerine jelly in the form of a thin film, was inserted through the slide slit, used for trapping the pollen grains. At the end of the sampling, the slide was removed by rotating the upper ring and with the help of suitable forceps. A sample of 2 mm × 14 mm was deposited on the glass slide as the sampler sucked air through the slit at the top and the particles were deposited on the glycerine jelly-coated micro slide in the form of a thin band. The exposed slides were mounted with DPX as a mountant by placing a 14 sq. mm microscopic coverslip and were allowed to dry for two to three days. The slides were scanned under a high-resolution binocular light microscope.

2.3. Preparation of Reference Pollen Slides

For evaluation of the aero palynological assemblage of an area, a decent knowledge of its surrounding flora and their pollen grains are of prime importance. The sources of airborne pollen in an area are mainly the local plants. As such, it is essential to identify all the plants growing in the area, enumerate them, and note their flowering period and mode of pollination. So, a detailed and systematic survey was carried out from January 2013 to December 2014 at each of the study sites, covering a 10 km radius of the sampling site.

In Santiniketan, *Saccharum spontaneum* is growing in abundance and flowers during the entire autumn time abundantly, thus showing the aerial dominancy of their pollen during this period. In addition, this pollen has not been characterized yet as a potential allergen in West Bengal (Figure 2).

Figure 2. Versatile anthers of *Saccharum spontaneum*.

2.4. Pollen Extract Preparation

Mature anthers were collected from SS grass growing in different areas of Santiniketan and Kolkata, West Bengal. The anthers were shaded by shaking the whole inflorescences carefully. The brown to bright yellow-colored anthers were collected and dried over a hot plate for 3–4 days (Figure 2). The dried materials were ground and passed through different grades of sieves to obtain pure pollens with fewer contaminants (>95% pollen, <5% anther fragments). Pollen purity was assessed by microscopic analysis. Pollens were defatted using peroxide-free diethyl ether before extraction of antigens [60]. Crude pollen extract of *Saccharum spontaneum* was prepared in 100 mM ammonium bicarbonate buffer (pH 8.0) as described by Ghosal et al. 2016 [61]. The crude extract was dialyzed using tube-o-dialyzer (membrane of MW cut off 8000 Da) (G-Bioscience, St. Louis, MO, USA) against the same extraction buffer for 16 h at 4 °C with frequent changes of buffer and finally passed through a 0.22 µm Millipore filter paper using syringe filter (Millipore Corp., Bedford, MA, USA). The aliquots of the extract were lyophilized and stored at −20 °C. The protein contents were estimated according to the Bradford method using the BioRadTM protein estimation kit (BioRad, Hercules, CA, USA).

2.5. Fractionation of Pollen Antigen

The whole antigenic extract was fractionated using ammonium sulphate (AS) cut in a range of 0–90% cut and intermittently centrifuged at 10,500× *g* for 15 min at 4 °C. The fractionated precipitation after being dissolved in extraction buffer was dialyzed to remove the traces of ammonia and then stored at −20 °C [62–64]. The protein content of the fraction was determined using Bradford reagent (Bio-Rad^{TM} Protein Estimation Kit, Hercules, CA, USA) following the manufacturer's manual.

2.6. Concentrating the Pollen Proteins for Protein Profiling

The crude extracted pollen antigens were concentrated using a Universal protein precipitating agent (UPPA) kit (GBioscience, A Geno Technology Inc., St. Louis, MO, USA), and protein precipitation was performed following the manufacturer's protocol followed by repeated acetone wash and was then reconstituted in SDS loading buffer for running in 12% SDS-PAGE [63].

2.7. Skin Prick Tests and Sera Collection

Allergic patients visiting an allergy diagnostic clinic in Kolkata, West Bengal were tested with crude antigenic pollen extracts (1:10 w/v) of *Saccharum spontaneum* mixed with 10% glycerine at a final concentration of protein 200 ng/µL. A total of 134 respiratory allergy patients (aged 12–60 years) were tested according to the method of Shivpuri 1962 with little modification (Sircar et al. 2015) [64,65]. Phosphate-buffered saline (PBS) and histamine diphosphate (100 µg/mL) were used as negative and positive controls, respectively. SPT was also carried out on 3 non-allergic healthy volunteers and the sera were collected, for negative control. Prior written consent of all the patients was taken. According to international guidelines, cutaneous positivity was defined as a mean wheal diameter $\geq$3 mm compared to negative control with no wheal and flair [66]. The reaction was graded from +1 to +4 level (+1 = erythema, 20 mm in diameter, +2 = wheal and erythema >20 mm in diameter, +3 = wheal >3 mm and erythema, +4 = wheal and pseudopod, erythema) according to Stytis et al. [67]. Corticosteroids and antihistamines were prohibited for 48 h before SPT to avoid reduction sensitivity of SPT. Patients showing a positive cutaneous response against *Saccharum spontaneum* pollen antigens were selected, and 2 mL of peripheral blood was collected for immunological studies. The total IgE in the sera was quantified by using a commercial Total IgE quantification kit (Pathozyme) following the manufacturer's protocol.

The exclusion criteria of SPT were perennial or severe asthma, pregnancy or lactation, malignancy, smoking, chronic infection, and other severe systemic diseases during skin testing or sera collection. A detailed history including age, sex, family history, onset, and duration of symptoms was recorded. Clinical history, symptoms of allergic manifestation, and a demographic profile of the patients were surveyed through a detailed standardized questionnaire [28,30,58,61].

2.8. Specific IgE Estimation Using Enzyme Linked Immunosorbent Assay (ELISA)

Direct ELISAs were performed to measure the specific IgE levels to SS pollen allergens in individual patient serum samples by the method of Engvall and Pearlman [68] with a partial modification of Bodinier et al. [69]. Briefly, 100 ng/µL pollen antigen (pH 8.0) was added per well of multisorbent ELISA plates (Nunc, Thermo, Waltham, MA, USA) and incubated overnight at 4 °C. The plate was washed three times with 100 µL phosphatebuffered saline (PBS-Tween 20-0.1 M phosphate-buffered saline pH 7.4 with 0.05% Tween 20). After blocking with 3% BSA (Bovine Serum Albumin), the ELISA plate was washed again with PBS-Tween 20. For estimation of specific IgE, wells were incubated with 50 µL of individual patients' sera diluted (1:10 v/v) with blocking solution (PBS-T-BSA) at 37 °C for 16 hrs. Further washing with PBS-T was followed by incubation with 100 µL of 'Monoclonal Anti-human IgE Clone GE-1-alkaline phosphatase conjugate as a secondary antibody (Sigma Chemical Co., Burlington, MA, USA) at 1:1000 dilution with blocking solution at 37 °C for 3 h. Following washing, 100 µL of para-nitro phenyl phosphate (pNPP) (Sigma Chemical, St. Louis, MO, USA) liquid substrate for ELISA was added to each well. The reaction was stopped after 30 min by adding 50 µL of 3 N NaOH to each well and the absorbance was measured at 405 nm using an ELISA reader (Multiscan-lab system, Helsinki, Finland). The mean OD405 values of all the healthy subjects were labeled as 'N' and the average OD_{405} value of the replica for each patient's serum was designated as 'P'. The ratio between P and N was considered as the level of serum-specific IgE as described by Sircar et al. [70]. For a particular serum, a P/N ration (patient vs. non-allergic subjects)

greater than 3.5 was considered as in vitro "positive" with markedly elevated levels of specific IgE against the study antigen.

2.9. Statistical Analysis

Regression analysis was used to fit regression equations for the total serum IgE using the specific IgE value for each type of pollen antigen as a covariate. Box plots were used to find the distribution of specific IgE levels over different groups based on the grade of SPT score (+1 to +4). From boxplots, along with upper and lower quartiles, median values were found. All statistical analysis and computation was undertaken with R studio ver. 3.2.2 where p-values < 0.05 were considered to be statistically significant.

2.10. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Periodic Acid Schiff (PAS) Staining

SDS-PAGE was carried out with the Laemmli (1970) [71] buffer system [0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4] using Mini-vertical gel electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). Total protein, AS precipitated fractions, and UPPA-treated sample protein were used for electrophoresis. Protein bands were detected by staining with Coomassie brilliant blue R-250 (CBB). The molecular mass of the protein bands was calculated by calibrating with broad range marker protein [Sigma-Aldrich™, Burlington, MA, USA]. The gel was documented using Gel Doc 1000 (Bio-Rad, USA) using the MOLECULAR ANALYST software (Bio-Rad, Hercules, CA, USA).

Glycoprotein fractions in the total protein were detected using a Glycoprotein Staining Kit (G Bioscience) following the manufacturer's protocol. The kit uses the Periodic Acid-Schiff (PAS) method for revealing carbohydrate moiety [60].

3. Results

3.1. Composition and Relative Abundance of Airborne Pollen

The aero palynological investigation revealed a wide range of pollen spectra in the atmosphere of Santiniketan in two successive years. A total of 63 pollen types were identified belonging to 38 families of angiosperms and 1 Gymnosperm from Santiniketan during the entire 2-year study period though a few pollen types remained unidentified.

Poaceae (grasses) alone contributed the maximum pollen load, followed by *Acacia* sp., while the minimum amount of pollen was contributed by *Zizyphus* sp. Supplementary Figure S2.

3.2. Seasonal Variation and Pollen Calendar

Five more or less well-defined seasons, i.e., autumn (October–November), winter (December–February), spring (March–April), summer (May–June) and monsoon (July–September) were demarcated in the study sites. The total number of pollen grains recorded in different months demonstrated a relatively significant variance (Supplementary Figure S2). The changes in the frequency of palynomorphs were probably related to the length of the flowering period, pollen productivity, and their extent of dispersal. The flowering period of the majority of the trees began in the later part of winter and extended until the early monsoon period. Each pollen grain type had a peak month of occurrence.

3.3. Protein Concentration of the Crude Antigenic Extract

The total soluble protein concentration of the crude antigenic extract of SS pollen was found to be 8–10 µg per gram of pollen. The SS antigen possessed a higher amount of protein in the ammonium sulphate fraction (90% fraction) than in the UPPA I/UPPA II treated crude extract.

3.4. Sensitization to SS Pollen

To evaluate the allergenic potential of SS pollen, its antigenic extract was tested in-vivo positive cutaneous skin prick test (SPT) on 134 patients (n = 134; age range:

12–60 years) with respiratory allergies having different symptoms such as early spring hay fever, allergic rhinitis, and seasonal allergic conjunctivitis. The results of intradermal SPT reaction towards Kans grass pollen antigenic extract conducted on the respiratory allergic patients at two different centers of the allergy diagnostic clinic in Kolkata, West Bengal are presented in Figure 3. Among them, 94 atopic patients exhibited positive skin reactions by SPT accounting for 70.14% to SS pollen allergen. Of these 65 subjects showing marked positivity, i.e., ≥2+ grade in SPT were considered to be clinically significant. Among them, +3/+4 grade of positivity was found to be most predominant followed by +2. Only 15 patients with marked positive reactions in SPT agreed to donate blood and the serum samples collected were subjected to specific IgE estimation by indirect ELISA [59,72]. Most of the patients tested against common inhalant grass and other allergens showed polysensitization (Table 1). Since it was not possible to directly include hospitalized asthma patients for skin testing, we performed skin tests on outpatients who visited the clinic for rhinitis and asthma.

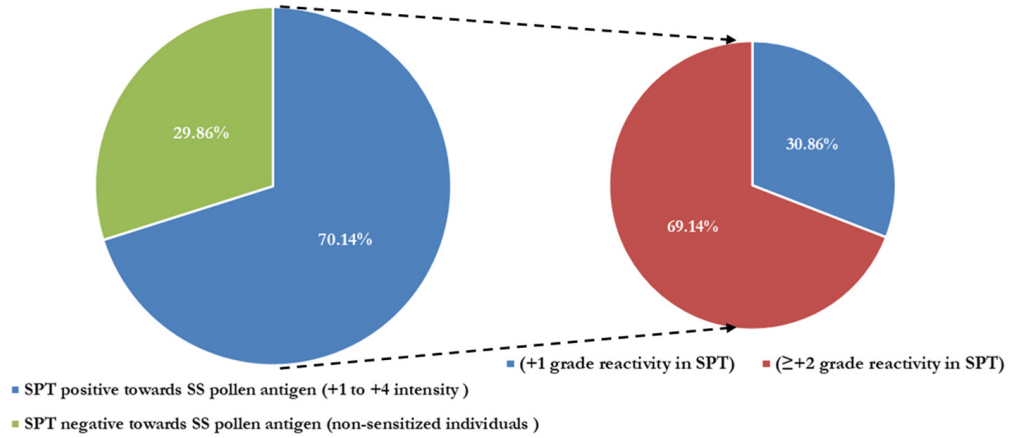

Figure 3. Results of skin prick (SPT) with SS antigenic extract (1:10 *w*/*v*) at different diagnostic clinics on 134 patients (*n* = 134; age range: 12–60 years).

Table 1. Result and fitted equation of regression analysis using bi-variate regression showing the relationship between Specific IgE as a predictive variable and Total IgE as the response variable.

	Significant Co-Variance	Coefficient	Std. Error	t-Value	*p*-Value	Adjusted R^2 Value
Saccharum spontaneum	(Intercept)	144.25	33.81	4.266	<0.001 *	
	Specific IgE	157.68	10.68	14.760	<0.001 *	0.7861
	Equation: Total IgE = 144.25 + 157.68 × Specific IgE value					

* means the correlation is statistically significant.

The other tested allergens for determination of their co-sensitization with SS antigens included 13 tree pollen types (*Acacia auriculiformis*, *Areca catechu*, *Azadirachta indica*, *Borassus flabellifer*, *Carica papaya*, *Cassia fistula*, *Syzigium cumini*, *Cocos nucifera*, *Delonix regia*, *Eucalyptus citriodora*, *Peltophorum pterocarpum*, *Phoenix sylvestris*, *Phyllanthus emblica*), three shrub pollen grains (*Cassia sophera*, *Cycas circinalis*, *Solanum symbriifolium*), two grass (Poaceae) pollen grains (*Saccharum spontaenum*, *Cynodon dactylon*), six fungal spores types (*Alternaria alternata*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Rhizopus nigricans*, *Pinicillium oxalicum*), and five foods (prawn, brinjal, pineapple, egg, soyabean) (Table 1).

The specific IgE values in the sera of 15 SPT-positive patients (2+ and above) from different places of West Bengal, as compared to control sera showing a negative skin test to SS extract, are presented in Table 1 and Figure 2. About 69.14% of the patients eliciting (2+ and above) marked positivity had shown elevated specific IgE titer in their sera. Serum samples from nine patients showed a P/N value of more than 3.5 with a high titer in ELISA and skin reactivity with various symptoms such as seasonal rhino-conjunctivitis,

breathlessness, nasal blocking, allergic rhinitis, bronchial asthma (or their combination) were selected for in vitro immuno-biochemical analysis (Figure 4).

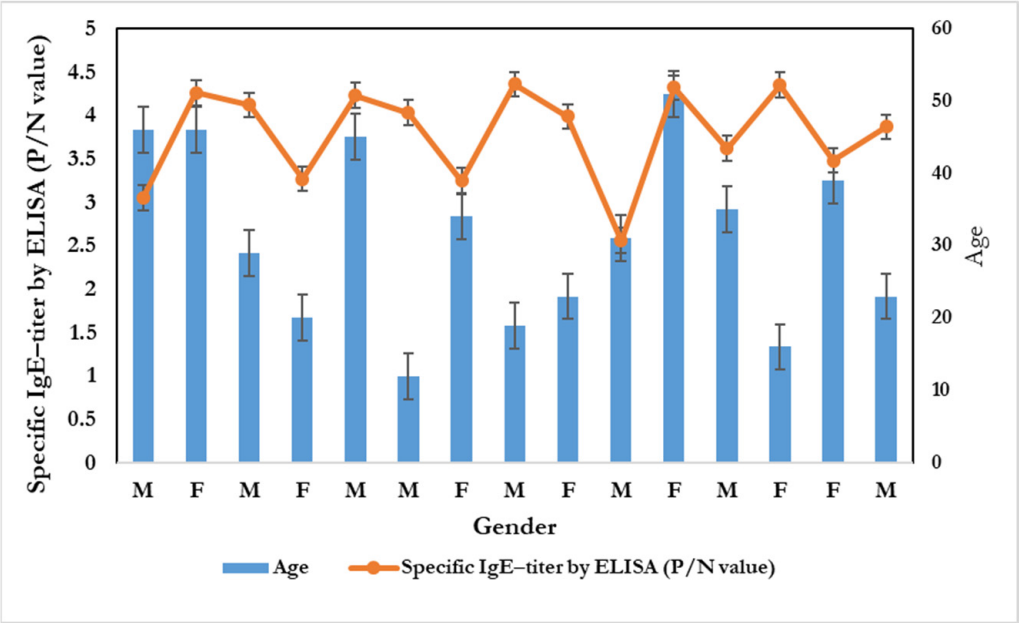

Figure 4. Results of specific IgE (P/N) with individual patients' sera against SS allergenic pollen extracts. (P/N represents mean ± SD of triplicate determination).

The questionnaire survey and case history of patients provided us with some valuable information regarding the allergic manifestation of atopic patients [41]. The epidemiology of allergic diseases is a complex phenomenon that depends on factors such as genetic predisposition, exposure to air pollution, and exposure to allergens from pollen grains which are unambiguously associated with symptomology and exacerbation of the human respiratory allergic disease [59,65]. The 21–40 years age group of patients used to visit the allergy clinic more frequently with various allergic symptoms during the study period, followed by the age group of 41–60 years, <20 years and the least allergic manifestation was shown in the patients aged more than 60 years, due to immunosuppression. From the clinical survey report, it was found that a significant part of the atopic population was suffering from SS-induced allergic symptoms namely cough, bronchial asthma, allergic rhinitis, allergic conjunctivitis, and skin allergy.

3.5. Statistical Analysis (Correlation–Regression Analysis and Box-Whisker Plot)

3.5.1. Correlation Analysis

Scatter plots for each pollen allergen have demonstrated that total serum IgE and the symptom score are dependent variables, as in most cases, total IgE increases when the symptom score increases with some exceptions in some data points (Figure 5). Pearson product-moment correlation analysis showed that total IgE is positively and significantly correlated with symptom scores with corresponding $p < 0.05$ for all the pollen allergens.

3.5.2. Box-Whisker Plot

The Box-whisker plots of Specific IgE levels over different categories of SPT scores showed that the median value for SPT grade 4 is at a maximum compared to other categories (Figure 5). Clinically speaking, if the specific IgE level was higher for any individual, the SPT grade would be higher too. It was also found that most of the specific IgE values belonged to the second and third categories of the SPT grade. The Box-whisker plots of Specific IgE levels over different categories of SPT scores showed that the median value for SPT grade 4 is at a maximum compared to other categories (Figure 6). Clinically speaking,

if the specific IgE level was higher for any individual, the SPT grade would be higher too. It was also found that most of the specific IgE values belonged to the second and third categories of the SPT grade.

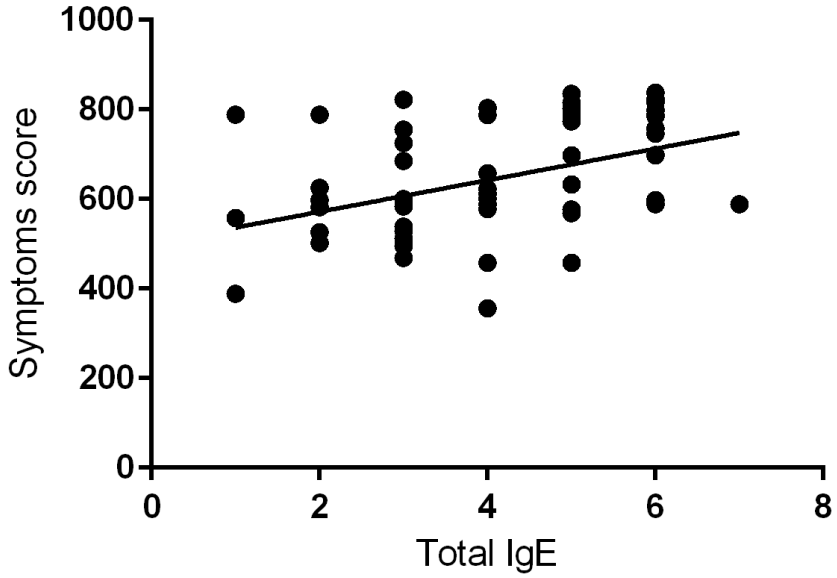

Figure 5. Scatter plot showing the relationship between total IgE value and symptom score showing a trendline to show the pattern (p value < 0.0008).

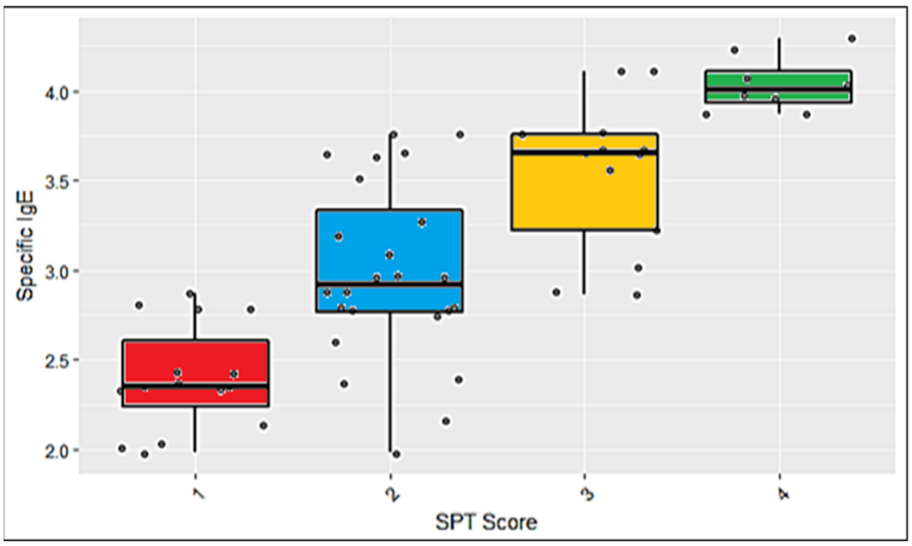

Figure 6. Box-Whisker plot for specific IgE levels over different categories of SPT grade.

The median value for SPT grade 4 is at a maximum compared to other grades in response to antigenic extracts of *Saccharum spontaneum*. Clinically speaking, in this case, specific IgE levels did not always reflect the SPT score (Figure 6).

Likewise, for *Saccharum spontaneum*, we calculated the adjusted R^2 value was 0.7861 which means the above regression equation and graph can explain almost 78% of the variability. So, the prediction of patients' total IgE would be 78% accurate which means that the model is quite fit (Table 1). The fitted regression line for total IgE and Specific IgE was plotted in Figure 7.

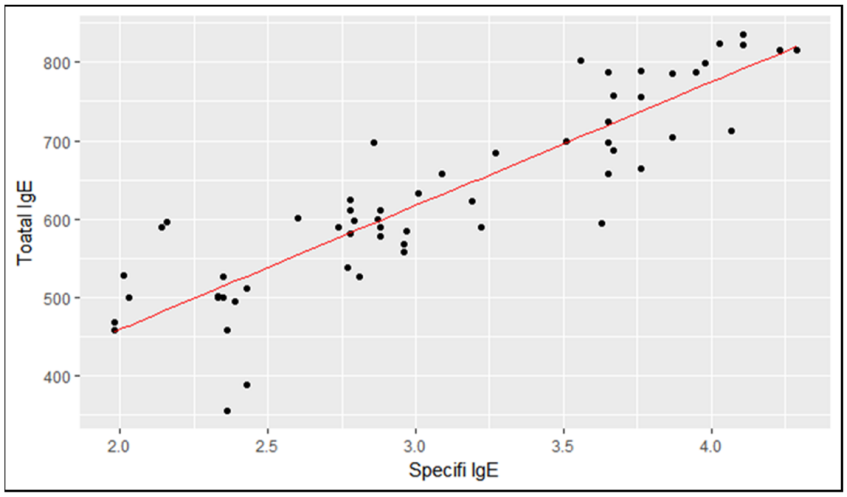

Figure 7. Fitted line plots (straight line) displaying the results from simple regression along with the observed values (dots), which is one predictor variable (specific IgE) and the total IgE.

3.6. Allergen Profile of SS Grass Pollen

3.6.1. SDS-PAGE

The soluble crude extract (UPPA treated) of *Saccharum spontaneum* pollen antigen separated into more than 15 CBB-stained bands in the molecular weight (MW) range of 14.4 to 116 kDa (Figure 8A) on 12% SDS-PAGE. Proteins of MW 116.0, 106.4, 91.2, 72.3, 62.2, 53.8, 42.6, 38.0, 34.3, 33.8, 25.3, 21.4, 20.4, 19.6, and 17.5 kDa, respectively, were stained prominently with CBB in concentrated extract, while all these protein bands were found to be distinct in fraction Fr.90 when stained in CBB, except for the 25.3 kDa band (Figure 8A). A 20.4 kDa protein fraction was the most prominent among all the visible bands. So, two sets (UPPA-treated antigen and 90% AS fraction) of SS extract produced nearly the same pattern on SDS-PAGE (50, 51).

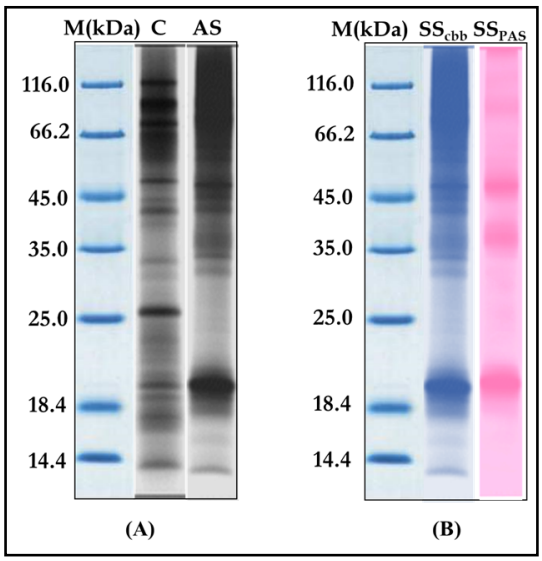

Figure 8. A 12% SDS-PAGE of crude (concentrated) and ammonium sulphate fraction pollen extracts of *Saccharum spontaneum* [M = Molecular Marker, Crude = UPPA treated Crude extract, AS: 90% ammonium sulphate fraction] (**A**) (CBB-250 staining); (**B**) (Periodic Acid Schiff (PAS) staining) [Lane SS_{cbb} = SS antigenic extract stained in Coomassie Brilliant Blue 250; Lane SS_{PAS} = SS antigenic extract stained in Periodic Acid Schiff staining].

3.6.2. PAS Staining

It was previously reported in various research articles [73–77] that Poaceae (grass) pollen allergens were glycoproteins in nature. Hence, we have performed the PAS staining of the SDS-PAGE gel as well as the protein-loaded PVDF membrane of *Saccharum spontaneum*. In PAS staining, some high molecular weight proteins along with a few low molecular weight proteins were found to contain glycan moieties as these appeared as magenta bands while others were not, indicating that no carbohydrate part was associated with them (Figure 8B).

4. Discussion and Conclusions

The pre-requisite for executing an aerobiological survey is the selection of a sampler. The sample techniques work on the principles of gravity, impaction, or suction [36]. The Burkard personal slide sampler was employed in this work to analyze the monthly and seasonal periodicities of airborne pollen grains in the specified study location (Santiniketan, West Bengal, India). This sampler has certain advantages because it is portable and transportable and can be powered by either current or batteries. Because of its portability, it can be simply transported from one experimental site to another, allowing us to sample in multiple areas. One of the main goals of aerobiological observations is to define the pollen spectrum in a given region. Our aerobiological monitoring revealed a wide range of atmospheric pollen spectra, both quantitatively and qualitatively, in two successive years in the atmosphere of Santiniketan, which helped us to construct a comprehensive pollen calendar of this sub-urban region (Supplementary Figure S1). The significance of the pollen calendar lies in the fact that it provides a highly descriptive representation of the dynamics of aero pollen throughout the year of a particular locality [59,78]. Documentation of airborne pollen in this geographical zone of West Bengal may help in understanding the flowering behavior of local vegetation also. Poaceae, which produces anemophilous pollen, has proven to be the predominant pollen type in both of the years, earning it special consideration for allergic research. It is proposed that a study on the grass pollen of such regions and their daily pollen release can aid pollen allergy patients in preventing exposure to the allergen because Poaceae is a strong stenopalynous family [9,18,22,35,50–52,54,73,74,79].

To protect the public's health, it is crucial to have a thorough understanding of the pollen season's prevalence and intensity [8,9,11]. Every species that was captured in the sampler displayed variation in how the pollen season developed. Depending on the taxa, the consecutive phases (percentiles) of the pollen season are achieved at different times. Although sources of aeroallergen may arise even before a pollen season begins, the beginning of the pollen season is the most crucial time to forecast because it correlates with the onset of allergic symptoms in nearby residents [69,70]. The current study demonstrated that variations in geology, vegetation, and other climatic conditions cause differences in the airborne pollen spectrum in terms of quantitative and qualitative assemblages of pollen from place to place.

Our study is the first to report and identify the pollen of Kans grass (*Saccharum spontaneum*) as a potential aeroallergen from West Bengal, the eastern part of India. *Saccharum spontaneum* is seasonal grass surviving hot and humid tropical weather, therefore they grow all over India, especially in the eastern, southern, and western parts. Due to its morphological appearance and versatile nature of anthers (Figure 2), Kans grass liberated a large number of tiny pollen grains into the atmosphere during its seasonal blooming. This pollen was found to cause sensitization among 70.14% of allergic patients (Figure 3), which was an alarming situation for the atopic population, as it causes early spring hay fever, allergic rhinitis (seasonal), and intermittent or seasonal allergic conjunctivitis. Two years of consecutive biomonitoring and phenological studies established the pollinating period of the aforesaid grass species being within a range from August to October, considering the fact that the phenology was greatly influenced by meteorological and climatic factors [80]. The studies on flowering phenology help in the understanding of results of aerobiological investigations, and also, in a more accurate explanation of the co-occurrence of grass pollen

in the atmosphere [81,82]. The months of blooming season and flowering phenology of the eight selected grasses along with *Saccharum spontaneum* showed the co-presence of their pollen grains from July to October (Supplementary Figure S2). In the present study, the allergic potency and immunological characterization of Kans grass pollen have been taken for investigation, despite its seasonal presence in the air, as researchers have shown that allergic manifestations may be triggered even at low pollen concentrations [48,49,83].

Sensitization to SS pollen among a cohort of the Indian population has been evaluated in the present study by the in vivo skin prick test and in vitro immunoscreening in a panel of nine sera having different symptoms of respiratory allergic manifestation. The results of SPT and ELISA demonstrate the allergic potential of pollen of SS on polysensitized patients attending the allergy clinic. Polysensitization is more prevalent than monosensitization in the general population as suggested in various surveys. The soluble crude extract of *Saccharum spontaneum* pollen antigen resolved into more than 15 distinct protein bands ranging from 14.4 to 116 kDa. These results with SS pollen protein components resolved in SDS-PAGE widen the field of potential future research to identify the specific IgE binding protein or any cross-reactive protein between the phylogenetically related species. Other Poaceae members have also been the subject of investigations on cross-reactivity and allergen characterization. However, in the former studies, such high molecular weight immunoreactive proteins have not been reported from the grass pollen antigen. Four protein components of MW 19.5, 38.0, 53.8, and 91.2 kDa were detected to be glycosylated though the precise nature of these glycoprotein allergens needs to be characterized. Cross-reactivity of carbohydrate moieties has also been reported by other researchers as well [84–86].

Correlation coefficient values were found to be high, positive, and statistically significant with the allergic patients. Regression analysis for the pollen antigen for estimating the total IgE value of a patient's sera from their specific IgE value was a novel approach by our study (54). For each of the pollen antigens, we have fitted a regression equation using specific IgE as the predictive and dependent variables. We also have mentioned the adjusted R2 value which describes the goodness of fit of all the models. In clinical practice, atopic patients, however, frequently show sensitization towards multiple sets of allergens, as we observed in Table 2. This may be due to cross-reaction or co-sensitization with different groups of allergens due to structural homology leading to the sharing of antigenic surfaces [5–8,18,20,30]. Pollen allergens from different grasses or taxonomically related families are known to have identical structural epitopes showing a high degree of cross-reactivity, such as the varied dose–response ELISA inhibition curves, suggested a close allergenic association with *Cenchrus*, *Imperata*, and *Pennisetum* [50]. In the same study, *Cenchrus* and *Imperata* have shown significant shared allergenicity with *Cynodon* extract, indicating structural homology [30]. A comprehensive allergenic relationship of *L. perenne*, *P. pratense*, *P. pratensis*, *Zea mays*, and *C. dactylon* pollen have been established by immunoblot inhibition with *I. cylindrica* pollen extract against allergic patients' sera [51]. Likewise, pollen of *Cynodon*, *Imperata*, *Pennisetum*, and *Sorghum* have shown allergic cross-reactivity with *C. ciliaris* pollen extract [54]. The discovery of these new pollen allergens may aid clinicians in the diagnosis, avoidance, and treatment of patients who are allergic to these pollen kinds. The phenology and aerial prevalence of the chosen pollen revealed a higher frequency of atmospheric presence over a long period of time, which may pose a possible threat to sensitize the atopic population due to the cross-reactive phenomenon.

In conclusion, this information on shared IgE reactive allergens is anticipated to reduce the number of allergens required for diagnosis or immunotherapy of respiratory allergic disorders [87]. The knowledge acquired from the present work on cross-reactive grass pollen allergens and their clinical implications during the management of atopic patients will provide a new prospect in allergy illnesses.

Table 2. Clinical characteristics of the 15 *Saccharum spontaneum* pollen-sensitized patients.

S1. No	Age/Sex	Symptoms/Clinical History *	Grades of SPT **	Specific IgE-Titer by ELISA (P/N Value)	Established Co-Sensitization***
P1	46/M	C, S	++	3.05	LS, SC, CD, CC, AFu, RH
P2	46/F	C, S, B, SK	+++	4.26	AC, SS, CP, PE, AAl, PO
P3	29/M	S, B, AC, NB	+++	4.12	PS, PE, CO, CD, AF
P4	20/F	S, NB	++	3.27	PP, CO, CD, AAl, RN
P5	45/M	S, AC, SK, NB	++++	4.23	AI, PP, SS, CD, CO, AN
P6	12/M	S, AC, SK, NB	+++	4.03	SS, CF, AF, AAl, AF
P7	34/F	S, B, AC, SK	++	3.25	AA, SS, SC, CD, AN, PO
P8	19/M	B, NB, S	+++	4.36	AI, SC, CD, AAl, RH
P9	23/F	S, B, AC, NB	++++	3.99	CF, SC, CD, AFu, RH
P10	31/M	C, NB, AC	+++	2.56	AI, PP, SS, CD, SC, AN,
P11	51/F	C, NB, S	+++	4.32	PS, PE, SC, CD, AF
P12	35/M	C, B, AC, SK, NB	+++	3.62	BF, CN, CS, SC, CD
P13	16/F	B, AC, NB	+++	4.35	BF, CP, CS, SC, CD
P14	39/F	C, S, B, AC, NB	++	3.48	AI, PP, SS, IC, SC, AN
P15	23/M	C, S, BA, AC, SK	++++	3.87	
Healthy Control patients					
		C1	–	0.59	No
		C2	–	0.72	No
		C3	–	0.56	No

* Symptoms: Cough (C), Breathlessness (B), Allergic conjunctivitis (AC), Sneezing (S), Nasal Blocking (NB), and Skin Rash (SR). ** The grading scale of positive skin prick reactions is as follows: negative if wheal diameter is <3 mm, +1 if wheal diameter is 3–5 mm, +2 if wheal diameter is >6 mm, +3 if wheal diameter is >6 mm, with one or two small pseudopods, and +4 is any reaction that is more pronounced than +3. *** Aeroallergens: AA—*Acacia auriculiformis*, AC—*Areca catechu*, AI—*Azadirachta indica*, BF—*Borassus flabellifer*, CP—*Carica papaya*, CF—*Cassia fistula*, SC—*Syzigium cumini*, CN—*Cocos nucifera*, PP—*Peltophorum pterocarpum*, PS—*Phoenix sylvestris*, PE—*Phyllanthus emblica*, CS—*Cassia sophera*, CC—*Cycas circinalis*, SS—*Solanum symbriifolium*, CD—*Cynodon dactylon*, AAl—*Alternaria alternata*, AF—*Aspergillus flavus*, AFu—*Aspergillus fumigatus*, AN—*Aspergillus niger*, RN—*Rhizopus nigricans*, PO—*Pinicillium oxalicum*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/aerobiology1010004/s1, Figure S1: Pollen calendar of Santiniketan; Figure S2: Flowering phenology of nine identified grasses growing around; Figure S3: Pollen morphology of nine types of grasses identified.

Author Contributions: All the authors had equal contribution in the conceptualization, investigation, methodology, and the experimental results discussion. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported financially by the departmental fund of the Environmental Biology Laboratory, Department of Botany, Visva Bharati, Santiniketan and Department of Botany, Midnapore College (Autonomous).

Institutional Review Board Statement: Ethical review and approval were waived for this study due to the REASON that the clinical part was done on the regular visiting patients by the doctor/clinician in their diagnostic centers.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Acknowledgments: I would like to modify this acknowledgment part. The new statement will be Authors of this study are thankful to Swati Gupta-Bhattacharya, Bose Institute, Kolkata, for allowing me to perform some of the clinical and immunological experiments in their laboratory. We express our gratitude to Arindom Chakraborty, Dept. of Statistics, Visva Bharati, Santiniketan for all the in-depth statistical analysis of aerobiological and clinical data.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sambrook, J.; Fritsh, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: New York, NY, USA, 1989.
2. Martínez-Bracero, M.; Alca´zar, P.; Diaz de la Guardia, C.; Gonza´lez-Minero, F.J.; Ruiz, L.; Trigo Pe´rez, M.M.; Galán, C. Pollen calendars: A guide to common airborne pollen in Andalusia. *Aerobiologia* **2015**, *31*, 549–557. [CrossRef]
3. Martin, B.G.; Mansfiels, L.E.; Nelson, H.S. Cross allergenicity among the grasses. *Ann. Allergy* **1985**, *54*, 99–104.
4. Mabberley, D.J. *The Plant Book*; Cambridge University Press: Cambridge, UK, 1987.
5. Ruden, S.; Steinman, H. *Grass Pollens. Allergy Which Allergens?* Pharmacia Diagnostics: Uppsala, Sweden, 2004.
6. Kleine-Tebbe, J.; Davies, J. Grass Pollen Allergens. In *Global Atlas of Allergy*; Section A—Allergy—From Genetics to Mechanisms; European Academy of Allergy and Clinical Immunology: Zurich, Switzerland, 2014; pp. 22–27.
7. Johnson, P.; Marsh, D.G. "Isoallergens" from rye grass pollen. *Nature* **1965**, *206*, 935–937. [CrossRef]
8. D'Amato, G.; Spieksma, F.T.M.; Liccardi, G.; Jäger, S.; Russo, M.; Kontoufili, K.; Nikkels, H.; Wüthrich, B.; Bonini, S. Pollen-related allergy in Europe. *Allergy* **1998**, *53*, 567–578. [CrossRef]
9. Suphioglu, C. What are the important allergens in grass pollen that are linked to human allergic disease? *Clin. Exp. Allergy* **2000**, *30*, 1335–1341. [CrossRef] [PubMed]
10. Andersson, K.; Lidholm, J. Characteristics and immunobiology of grass pollen allergens. *Int. Arch. Allergy Immunol* **2003**, *130*, 87–107. [CrossRef] [PubMed]
11. Mohapatra, S.S.; Lockey, R.F.; Shirley, S. Immunobiology of grass pollen allergens. *Curr. Allergy Asthma Rep.* **2005**, *5*, 381–387. [CrossRef] [PubMed]
12. Türe, C. Allergenic airborne poaceae (grass) pollen around public transportation centers in Eskişehir, Turkey. *South-West. J. Hortic. Biol. Environ.* **2016**, *7*, 1–14.
13. Adams, E.; Perkins, W.E.; Estes, J.R. Pollination systems in *Paspalum dilatatum* Poir. (Poaceae): An example of insect pollination in a temperate grass. *Am. J. Bot.* **1981**, *68*, 389–394. [CrossRef]
14. Lewis, W.H.; Vinay, P.; Zenger, V.E. *Airborne and Allergenic Pollen of North America*; Johns Hopkins University Press: Baltimore, MD, USA, 1983.
15. Frisk, C.A.; Apangu, G.P.; Petch, G.M.; Adams-Groom, B.; Skjøth, C.A. Atmospheric transport reveals grass pollen dispersion distances. *Sci. Total Environ.* **2022**, *814*, 152806. [CrossRef]
16. Severova, E.; Kopylov-Guskov, Y.; Selezneva, Y.; Karaseva, V.; Yadav, S.R.; Sokoloff, D. Pollen Production of Selected Grass Species in Russia and India at the Levels of Anther, Flower and Inflorescence. *Plants* **2022**, *11*, 285. [CrossRef] [PubMed]
17. Connor, H.E. *Grass Systematics and Evolution*; Smithsonian Institution Press: Washington, DC, USA, 1986.
18. Garcia-Mozo, H. Poaceae pollen as the leading aeroallergen worldwide: A Review. *Allergy* **2017**, *72*, 1849–1858. [CrossRef]
19. Petersen, A.; Suck, R.; Hagen, S.; Cromwell, O.; Fiebig, H.; Becker, W.M. Group 13 grass allergens: Structural variability between different grass species and analysis of proteolytic stability. *J. Allergy Clin. Immunol.* **2001**, *107*, 856–866. [CrossRef] [PubMed]
20. Esch, R.E. Grass pollen allergens. *Clin. Allergy Immunol.* **2008**, *21*, 107–126. [PubMed]
21. Mathiesen, F.; Lowenstein, H. Group V allergens in grass pollens-II. An investigation of group V allergens in pollen from ten grasses. *Clin. Exp. Allergy* **1991**, *21*, 309–320. [CrossRef]
22. Kumar, L.; Sridhara, S.; Singh, B.; Gangal, S. Characterization of Cogon Grass (*Imperata cylindrica*) Pollen Extract and Preliminary Analysis of Grass Group 1, 4 and 5 Homologues Using Monoclonal Antibodies to Phleum pratense. *Int. Arch. Allergy Immunol.* **1998**, *117*, 174–179. [CrossRef]
23. Westritschnig, K.; Horak, F.; Swoboda, I.; Balic, N.; Spitzauer, S.; Kundi, M.; Fiebig, H.; Suck, R.; Cromwell, O.; Valenta, R. Different allergenic activity of grass pollen allergens revealed by skin testing. *Eur. J. Clin. Investig.* **2008**, *38*, 260–267. [CrossRef]
24. Kailaivasan, T.; Davies, J.M. The molecular allergology of subtropical grass pollen. *Mol. Immunol.* **2018**, *100*, 126–135. [CrossRef]
25. Erbas, B.; Chang, J.H.; Dharmage, S.; Ong, E.K.; Hyndman, R.; Newbigin, E.; Abramson, M. Do levels of airborne grass pollen influence asthma hospital admissions. *Clin. Exp. Allergy* **2007**, *37*, 1641–1647. [CrossRef]
26. Erbas, B.; Chang, J.H.; Newbigin, E.; Dhamarge, S. Modelling atmospheric concentrations of grass pollen using meteorological variables in Melbourne, Australia. *Int. J. Environ. Health Res.* **2007**, *17*, 361–368. [CrossRef]
27. Erbas, B.; Akram, M.; Dharmage, S.C.; Tham, R.; Dennekamp, M.; Newbigin, E.; Taylor, P.; Tang, M.L.; Abramson, M.J. The role of seasonal grass pollen on childhood asthma emergency department presentations. *Clin. Exp. Allergy* **2012**, *42*, 799–805. [CrossRef] [PubMed]
28. Basak, T.; Chakraborty, A.; Bhattacharya, K. Aerial pollen diversity of two bio-geographical zones of West Bengal and their clinical significance in allergic diseases. *Int. J. Pharm. Biol. Sci.* **2017**, *8*, 449–460.

29. Basak, T.; Chakraborty, A.; Bhattacharya, K. Investigation of aerial pollen diversity in Santiniketan, West Bengal and prediction of pollen concentration: A new statistical approach for forecasting of pollen season. *Indo Am. J. Pharm.* **2017**, *4*, 4576–4587.
30. Singh, B.P.; Sinha, S.; Arora, N. Cross reactive allergens: Status and their clinical implications. *Indian J. Allergy Asthma Immunol.* **2008**, *22*, 105–118.
31. Malik, P.; Singh, A.B.; Babu, C.R.; Gangal, S.V. Atmospheric concentration of pollen grains at human height. *Grana* **1991**, *30*, 129–135. [CrossRef]
32. Chakraborty, P.; Gupta Bhattacharya, S.; Chakraborty, C.; Lacey, J.; Chanda, S. Airborne allergenic pollen grains on a farm in West Bengal, India. *Grana* **1998**, *37*, 53–57. [CrossRef]
33. Basak, T.; Chakraborty, A.; Bhattacharya, K. Atmospheric pollen spectra of an historical french colony, Chandannagar, West Bengal, India. *Indian J. Aerobiol.* **2015**, *28*, 21–36.
34. Verma, J.; Singh, B.P.; Gangal, S.V.; Arora, N.; Sridhara, S. Purification and partial characterization of a 67-kD cross-reactive allergen from *Imperata cylindrica* pollen extract. *Int. Arch. Allergy Immunol.* **2000**, *122*, 251–256. [CrossRef]
35. Choi, Y.J.; Lee, K.S.; Oh, J.W. The Impact of Climate Change on Pollen Season and Allergic Sensitization to Pollens. *Immunol. Allergy Clin. N. Am.* **2021**, *41*, 97–109. [CrossRef]
36. Bhattacharya, K.; Sircar, G.; Dasgupta, A.; Gupta Bhattacharya, S. Spectrum of Allergens and Allergen Biology in India. *Int. Arch. Allergy Immunol.* **2018**, *177*, 219–237. [CrossRef]
37. Watson, L.; Dallwitz, M.J. The Grass Genera of the World: Descriptions, Illustrations, some regions of Turkey (Revıew). *Astım Allerji İmmünoloji* **1992**, *3*, 131–137.
38. Tripodi, S.; Frediani, T.; Lucarelli, S.; Macrì, F.; Pingitore, G.; Di Rienzo Businco, A.; Dondi, A.; Pansa, P.; Ragusa, G.; Asero, R.; et al. Molecular profiles of IgE to *Phleum pratense* in children with grass pollen allergy: Implications for specific immunotherapy. *J. Allergy Clin. Immunol.* **2012**, *129*, 834–839. [CrossRef] [PubMed]
39. Ghitarrini, S.; Pierboni, E.; Rondini, C.; Tedeschini, E.; Tovo, G.R.; Frenguelli, G.; Albertini, E. New biomolecular tools for aerobiological monitoring: Identification of major allergenic Poaceae species through fast real-time PCR. *Ecol. Evol.* **2018**, *8*, 3996–4010. [CrossRef] [PubMed]
40. Hanson, M.; Petch, G.; Ottosen, T.B.; Skjøth, C. Summer pollen flora in rural and urban central England dominated by nettle, ryegrass and other pollen missed by the national aerobiological network. *Aerobiologia* **2022**, *38*, 591–596. [CrossRef]
41. Burbach, G.J.; Heinzarling, L.M.; Edenharter, G.; Bachert, C.; Bindslev-Jensen, C.; Bonini, S.; Bousquet, J.; Bousquet-Rouanet, L.; Bousquet, P.J.; Bresciani, M.; et al. GA (2) LEN skin test study II: Clinical relevance of inhalt allergen sensitizations in Europe. *Allergy* **2009**, *64*, 1507–1515. [CrossRef]
42. Leon-Ruiz, E.; Alcazar, P.; Domínguez-Vilchez, E.; Galan, C. Study of Poaceae phenology in a Mediterranean climate. Which species contribute most to airborne pollen counts? *Aerobiologia* **2011**, *27*, 37–50. [CrossRef]
43. Cecchi, L. From pollen count to pollen potency: The molecular era of aerobiology. *Eur. Respir. J.* **2013**, *42*, 898–900. [CrossRef]
44. Freidhoff, L.R.; Ehrlich-Kautzky, E.; Grant, J.H.; Meyers, D.A.; Marsh, D.G. A study of the human immune response to *Lolium perenne* (rye) pollen and its components, Lol p I and Lol p II (rye I and rye II). I. Prevalence of reactivity to the allergens and correlations among skin test, IgE antibody, and IgG antibody data. *J. Allergy Clin. Immunol.* **1986**, *78*, 1190–1201. [CrossRef]
45. Weber, R.W. Patterns of pollen cross-allergenicity. *J. Allergy Clin. Immunol.* **2003**, *112*, 229–239. [CrossRef]
46. Malandain, H. Basic immunology, allergen prediction, and bioinformatics. *Allergy* **2004**, *59*, 1011–1012. [CrossRef]
47. Hrabina, M.; Peltre, G.; van Ree, R.; Moingeon, P. Grass pollen allergens. *Clin. Exp. Allergy Rev.* **2008**, *8*, 7–11. [CrossRef]
48. Singh, A.B.; Kumar, P. Climate change and allergic diseases: An overview. *Front. Allergy* **2022**, *3*, 964987. [CrossRef] [PubMed]
49. Buters, J.; Prank, M.; Sofiev, M.; Pusch, G.; Albertini, R.; Annesi-Maesano, I.; Antunes, C.; Behrendt, H.; Berger, U.; Brandao, R.; et al. HIALINE working group. Variation of the group 5 grass pollen allergen content of airborne pollen in relation to geographic location and time in season. *J. Allergy Clin. Immunol.* **2015**, *136*, 87–95. [CrossRef]
50. Sridhara, S.; Singh, B.P.; Kumar, L.; Verma, J.; Gaur, S.N.; Gangal, S.V. Antigenic and allergenic relationship among airborne grass pollens in India. *Ann. Allergy Asthma Immunol.* **1995**, *75*, 73–79. [PubMed]
51. Sridhara, S.; Kumar, L.; Verma, J.; Singh, B.P.; Gangal, S.V. Studies on *Cenchrus ciliaris* pollen extract and cross reactivity among tropical grasses of the family Poaceae. *Allergy Clin. Immunol. Int.* **2000**, *12*, 110–115. [CrossRef]
52. Sridhara, S.; Kumar, L.; Verma, J.; Arora, N.; Gaur, S.N.; Singh, B.P. Immunobiochemical characterization of *Sorghum vulgare* pollen allergens prevalent in tropical countries. *Indian J. Allergy Asthma Immunol.* **2002**, *16*, 33–39.
53. Liao, C.; Hou, X.; Wu, L.; Luo, W.; Zhang, H.; Sun, X.; Yu, Y.; Zhang, X.D.; Sun, B. Major Grass Pollen Allergen Components and Cross-Reactive Carbohydrate Determinants in Mugwort-Sensitized Child Patients with Allergic Respiratory Disease in Western China. *Front. Pediatr.* **2022**, *10*, 816354. [CrossRef]
54. Bijli, K.M.; Singh, B.P.; Sridhara, S.; Gaur, S.N.; Arora, N. An investigation of cross reactive allergens/antigens of *Imperata cylindrica* using western blotting and ELISA inhibition. *Allergy Clin. Immunol. Int.* **2003**, *15*, 62–67. [CrossRef]
55. Holm, L.; Doll, J.; Holm, E.; Pancho, J.; Herberger, J. *World Weeds: Natural Histories and Distribution*; John Wiley and Sons, Inc.: New York, NY, USA, 1997.
56. Frisk, C.A.; Adams-Groom, B.; Smith, M. Isolating the species element in grass pollen allergy: A review. *Sci. Total Environ.* **2023**, *883*, 163661. [CrossRef]
57. Boral, D.; Chatterjee, S.; Bhattacharya, K. The occurrence and allergising potential of airborne pollen in West Bengal, India. *Ann. Agric. Env. Med.* **2004**, *11*, 45–52.

58. Mandal, J.; Chakraborty, P.; Roy, I.; Chatterjee, S.; Gupta-Bhattacharya, S. Prevalence of allergenic pollen grains in the aerosol of the city of Calcutta, India: A two year study. *Aerobiologia* **2008**, *24*, 151–164. [CrossRef]
59. Basak, T.; Chakraborty, A.; Bhattacharya, K. Identification of airborne pollen allergens from two avenue trees of India, Int. *J. Environ. Health Res.* **2018**, *29*, 414–429. [CrossRef] [PubMed]
60. Ghosh, N.; Sircar, G.; Saha, B.; Pandey, N.; Gupta Bhattacharya, S. Search for Allergens from the Pollen Proteome of Sunflower (*Helianthus annuus* L.): A Major Sensitizer for Respiratory Allergy Patients. *PLoS ONE* **2015**, *10*, e0138992. [CrossRef]
61. Ghosal, K.; Saha, B.; Gupta Bhattacharya, S. Clinical and immuno-proteomic approach on *Lantana camara* pollen allergy-a major health hazard. *Allergy Asthma Clin. Immunol.* **2016**, *12*, 33. [CrossRef] [PubMed]
62. Mandal, J.; Roy, I.; Chatterjee, S.; Gupta Bhattacharya, S. Aerobiological and in vitro studies on pollen grains of two dominant avenue trees from Kolkata (Calcutta), India. *J. Investig. Allergol. Clin. Immunol.* **2008**, *18*, 22–30. [PubMed]
63. Saha, B.; Bhattacharya, S.G. Charting novel allergens from date palm pollen (*Phoenix sylvestris*) using homology driven proteomics. *J. Proteom.* **2017**, *165*, 1–10. [CrossRef] [PubMed]
64. Sircar, G.; Saha, B.; Mandal, R.S.; Pandey, N.; Saha, S.; Gupta Bhattacharya, S. Purification, cloning and immuno-biochemical characterization of a fungal aspartic protease allergen Rhi o 1 from the airborne mold *Rhizopus oryzae*. *PLoS ONE* **2015**, *10*, e0144547. [CrossRef]
65. Shivpuri, D.N. Comparative evaluation of the sensitivity of common methods of diagnostic antigen tests in the patients of respiratory allergy. *Indian J. Chest Dis.* **1962**, *4*, 102.
66. Dreborg, S.; Frew, A.J. (Eds.) Allergen standardization and skin tests. *Allergy* **1993**, *48*, 49–82. [CrossRef]
67. Stytis, D.P.; Stobo, J.D.; Fudenberg, H.; Wells, J.V. *Basic and Clinical Immunology*, 4th ed.; Lange Medical Publ., Maruzen Asia Pvt. Ltd.: Singapore, 1982.
68. Engvall, E.; Pearlman, P. Enzyme Linked Immuno Sorbent Assay (ELISA): Qualitative assay of immunoglobulin G. *Immunochemistry* **1971**, *8*, 871–879. [CrossRef]
69. Bodinier, M.; Brossard, C.; Triballeau, S.; Morisset, M.; Guerin-Marchand, C.; Pineau, F.; de Coppet, P.; Moneret-Vautrin, D.A.; Blanf, U.; Denery-Papini, S. Evaluation of an in vitro mast cell degranulation test in the context of food allergy to wheat. *Int. Arch. Allergy Appl. Immunol.* **2008**, *146*, 307–320. [CrossRef]
70. Sircar, G.; Chakrabarti, H.S.; Saha, B.; Gupta-Bhattacharya, S. Identification of aero-allergens from *Rhizopus oryzae*: An immuno-proteomic approach. *J. Proteom.* **2012**, *77*, 455–468. [CrossRef] [PubMed]
71. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685. [CrossRef] [PubMed]
72. Li, J.D.; Gu, J.Q.; Xu, Y.Y.; Cui, L.; Li, L.S.; Wang, Z.X.; Yin, J.; Guan, K. Serum IgE profiles in Chinese pollinosis patients with grass pollen sensitization. *World Allergy Organ. J.* **2022**, *15*, 100624. [CrossRef]
73. Ford, S.A.; Baldo, B.A. A re-examination of ryegrass (*Lolium perenne*) pollen allergens. *Int. Arch. Allergy Immunol.* **1986**, *81*, 193–203. [CrossRef]
74. Liao, C.; Liang, C.; Hu, H.; Luo, W.; Wu, G.; Huang, Z.; Wu, L.; Sun, B. Major Pollen Allergen Components and CCD Detection in Bermuda Grass Sensitized Patients in Guangzhou, China. *J. Asthma Allergy* **2020**, *13*, 615–623. [CrossRef] [PubMed]
75. Ball, T.; Edstrom, W.; Mauch, L. Gain of structure and IgE epitopes by eukaryotic expression of the major Timothy grass pollen allergen, Phl p 1. *FEBS J.* **2005**, *272*, 217–227. [CrossRef]
76. Bıçakçı, A.; Çelenk, S.; Altunoğlu, M.K.; Bilişik, A.; Canıtez, Y.; Malyer, H.; Sapan, N. Allergenic airborne Gramineae (Grass) pollen concentrations in Turkey. *Asthma Allergy Immunol.* **2009**, *7*, 90–99.
77. Zafred, D.; Nandy, A.; Pump, L.; Kahlert, H.; Keller, W. Crystal structure and immunologic characterization of the major grass pollen allergen Phl p 4. *J. Allergy Clin. Immunol.* **2013**, *132*, 696–703. [CrossRef]
78. Gala´n, C.; Smith, M.; Thibaudon, M.; Frenguelli, G.; Oteros, J.; Gehrig, R.; Berger, U.; Clot, B.; Brandao, R. Pollen monitoring: Minimum requirements and reproducibility of analysis. *Aerobiologia* **2014**, *30*, 385–395. [CrossRef]
79. Corti, V.; Cattaneo, A.; Bachi, A.; Rossi, R.E.; Monasterolo, G.; Paolucci, C.; Burastero, S.E.; Alessio, M. Identification of grass pollen allergens by two-dimensional gel electrophoresis and serological screening. *Proteomics* **2005**, *5*, 729–736. [CrossRef] [PubMed]
80. Gutierrez, H.F.; Medan, D. Flowering phenology, sexual expression and pollen production. *New Zealand J. Bot.* **2006**, *44*, 47–55. [CrossRef]
81. Kasprzyk, I. Flowering phenology and airborne pollen grains of chosen tree taxa in Rzeszów, SE Poland. *Aerobiologia* **2003**, *19*, 113–120. [CrossRef]
82. Kasprzyk, I.; Walanus, A. Flowering and airborne pollen—A novel statistical approach. *Acta Agrobot.* **2007**, *60*, 51–55. [CrossRef]
83. Fernandez-Gonzalez, D.; Gonzalez-Parrado, Z.; Vega-Maray, A.M.; Valencia- Barrera, R.M.; Camazon-Izquierdo, B.; De Nuntiis, P.; Mandrioli, P. *Platanus* pollen allergen, Pla a1: Quantification in the atmosphere and influence on a sensitizing population. *Clin. Exp. Allergy* **2010**, *40*, 1701–1708. [CrossRef]
84. Afferni, C.; Iacovacci, B.; Barletta, B.; Di Felice, G.; Tinghino, R.; Mari, A.; Pini, C. Role of carbohydrate moieties in IgE binding to allergenic components of *Cupressus arizonica* pollen extract. *Clin. Exp. Allergy* **1999**, *29*, 1087–1094. [CrossRef]
85. Ghosh, N.; Sircar, G.; Saha, B.; Pandey, N.; Bhattacharya, S.G. Data on mass spectrometry based identification of allergens from sunflower (*Helianthus annuus* L.) pollen proteome. *Data Brief.* **2016**, *7*, 735–739. [CrossRef]

86. Batard, T.; Sanjuan, A.; Denis, L.; Nguyen, H.; Montagut, A.; Sastre, J.; Rak, S.; Cuiné, J.F. Two grass pollen tablets commercially available for allergy immunotherapy display different IgE epitope repertoires. *Clin. Transl. Allergy* **2019**, *27*, 9–13. [CrossRef]
87. Gangl, K.; Niederberger, V.; Valenta, R. Multiple grass mixes as opposed to single grasses for allergen immunotherapy in allergic rhinitis. *Clin. Exp. Allergy* **2013**, *43*, 1202–1216. [CrossRef]