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ARTICLE

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Identification of airborne pollen allergens from two avenue trees of India

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ABSTRACT

An attempt has been made to detect airborne pollen of *Lagerstroemia speciosa* (LS) and *Spathodea campanulata* (SC) – two common avenue trees of India as potential sources of aeroallergens and also to identify the major IgE-reactive components present in them. The airborne pollen concentration was assessed using a Burkard sampler. A detailed questionnaire on clinical data of 1490 patients was recorded based on hospital data. We assessed the allergenicity of pollen by *in vivo* and *in vitro* tests. The correlation among meteorological factors, pollen seasons and allergenic potency of patients was assessed by multiple regression analysis. The sensitivity of patients to pollen antigens was highly correlated with pollen seasons. In SDS–PAGE, 15 protein bands were detected from LS pollen, while 14 bands from SC. The IgE-specific immunoblotting with patients' sera allergic to LS displayed five major allergens, while four major allergens were detected from SC. This would be the first report from India to prove the allergenic potentiality of airborne pollen of these two common avenue trees of India.

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Airborne pollen allergens; avenue trees of India; *Lagerstroemia speciosa*; *Spathodea campanulata*; immunoblot; multiple regression analysis

Introduction

Allergic diseases are of primary health concern across the world and have significantly affected the quality of life. Nearly 10–25% of the world's population is suffering from different allergic diseases (Anonymous 2000; Bhattacharya et al. 2018). Allergy to the pollen of flowering plants significantly impacts on the human health in many parts of the world (Vrtala et al. 1993; Puc 2003; Jianan et al. 2007; Bando et al. 2015; Sircar et al. 2015). Pollen allergy is mainly triggered by soluble proteins, glycoproteins or even a small peptide, which are present in the pollen wall (Chowdhury et al. 1998; Ghosh et al. 2015; Sircar et al. 2015; Saha and Gupta Bhattacharya 2017; Ghosal et al. 2016). Thus the detection of the source, isolation and characterization of allergy-causing reactive proteins are the primary and necessary tasks of aerobiologists and allergologist, to understand the molecular basis of allergy (Cresti and Tiezzi 1992; Pawankar et al. 2013; Saha et al. 2015; Sircar et al. 2016; Saha and Gupta Bhattacharya 2017; Bhattacharya et al. 2018).

Pollen contains many proteins for accomplishing its different stages of pollination and fertilizations, but only a small number of these exhibit allergenic properties (Knox and Heslop-Harrison 1970; Arnon and Van Regenmortel 1992; Vrtala et al. 1993; Giner 2002; Singaraja et al. 2008). The mode of transmission of pollen influences the chance and the intensity of exposure to allergens. The anemophilous pollen is imposing greater risk to human exposure than entomophilous pollen (Smart et al. 1979; Jimenez et al. 1994; Puc 2003). Hence, detailed knowledge on the aero-pollen flora of a given region and

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information on the immuno-biochemical nature of allergens present in airborne pollen would be helpful for the effective diagnosis of allergic patients (Boral et al. 1999, 2004; Hussain et al. 2013; García-Mozo 2017). The aerobiological surveys with reference to purification and characterization of pollen allergens in a few parts of India have been carried out by various workers (Banik and Chanda 1992; Gupta Bhattacharya et al. 1994; Chakraborty et al. 1998, 1999, 2005, 2009; Boral and Bhattacharya 2000; Mandal et al. 2008a; Chakrabarti et al. 2012; Hussain et al. 2012, 2014; Singh 2014), though many places have not been surveyed as yet.

The present study has been carried out in an unexplored region of West Bengal state, India, with an aim to identify the seasonal periodicity of airborne pollen of two avenue trees namely, *Lagerstroemia speciosa* (L.) Pers. and S*pathodea campanulata* P. Beauv and also to characterizing IgE reactive proteins present therein. This would be the first report on the allergenic potentiality of such avenue trees from India.

Materials and methods

Aerobiological sampling

An aerobiological survey was conducted for two consecutive years (January 2013 to December 2014) in Santiniketan (23.688°N 87.688°E), a small township centring a central university, 'Visva-Bharati' founded by the Nobel Laureate poet R.N. Tagore, situated about 150 km North-West of Kolkata megacity. The air sampling was performed by Burkard personal volumetric sampler (Burkard Manufacturing Co., UK) to record the seasonal periodicity of the airborne pollen of *L. speciosa* and *S. campanulata*. The sampler (air suction rate = 10 L/min) was placed about 1.5 m above ground level and was run for 10 min during three different time intervals: morning (09:30–10:30 hr), afternoon (12:30–13:30 hr) and evening (19:30–20:30 hr), thrice in a week. The exposed slides were mounted, scanned thoroughly by a high-resolution light microscope, and pollen grains were counted according to the guidelines of The British Aerobiology Federation (1995). We have combined the three counts (morning, afternoon and evening) to make a total counts and all the data were combined to express it as monthly counts. Thus, the hourly counts were averaged to obtain the mean concentration which in turn gave the monthly concentration expressed as pollen grains per cubic meter of air (Chakraborty et al. 1998).

Collection of meteorological data

The detailed meteorological data such as maximum and minimum temperature (°C), rainfall (mm), wind speed (km/h), relative humidity (%) were collected from the Sriniketan Meteorological station, situated about 2 km away from the sampling site.

Statistical analysis

The correlation between the meteorological factors and weekly pollen concentration was calculated using the Pearson non-parametric correlation coefficient analysis (Andersen 1980; Bricchi et al. 1992; Subiza et al. 1992). All computations were done in R studio (version 3.2.2) where values of $p < 0.05$ were considered to be statistically significant.

For the prediction of the allergy-related clinical visit of atopic patients and to find out the effects of *L. speciosa* and *S. campanulata* pollen count on them, Multiple regression analysis was also computed using R studio (ver. 3.2.2). The Multiple regression analysis was performed considering monthly pollen count of both the pollen, symptoms score of sensitive patients as predictive variables and number of individual sensitive patients who visited the clinical response variable (Ghosh et al. 2012). Because of uneven distribution of data Spearman's correlations were utilised for this statistical analysis. In statistical modelling, regression analysis is a statistical measure for assessing the relationships among variables.

Collection of pollen samples

The pollen samples were collected from mature anthers of the fresh flowers from *L. speciosa* and *S. campanulata* – two anemophylous plant – during their peak flowering period (Banik and Chanda 1992; Prakashkumar et al. 1998). Anthers were dried at 37°C, mildly crushed and passed through different sieves (150, 240, 300 and 400 mesh/cm^2) successively to remove the other floral impurities (Mandal et al. 2008b). The purity (> 95%) of the isolated pollen materials was confirmed under the microscope.

Antigenic extract preparation

To test allergic potency, pollen extracts were prepared from pure pollen samples following the method of Shivpuri (1962) and Sheldon et al. (1967). Pure pollen grains were defatted 3–4 times to remove the lipids and other insoluble pigments, using diethyl ether. Defatted pollens were then dried in vacuum desiccators containing $CaCl_2$ for 24–48 hrs and then stored in a dry airtight container at 4°C, till antigen extraction. The extract was prepared by incubating 10 mg of pollen in phosphate buffer saline (PBS) [0.1 M Na-phosphate, 150 mM NaCl, 50mM PMSF, 10mM EDTA, pH7.4] by continuous stirring at 4°C for 16 h in 1:10 (w/v) ratio (Hussain et al. 2012). After centrifugation at 14,000 rpm for 40 min, the clear supernatant was dialyzed in Tube-O-dialyser against PBS extraction buffer (pH 7.5) for 20 hrs. With frequent changes of buffer, the filtrate was passed through a 0.22 µm Millipore filter paper (Millipore Corp., Bedford, Massachusetts. the USA) using a syringe filter. The filtrate was then lyophilized and stored at −70°C in aliquots of known volume in sterile vials.

Fractionation of pollen antigen

The whole extract was fractionated using ammonium sulphate (AS) in three ranges such as Fr.30 [0–30% cut], Fr.60 [30–60% cut] and Fr.90 [60–90% cut] and intermittently centrifuged at 10,500 rpm for 15 min at 4°C. Each precipitated fraction after being dissolved in PBS was separately dialyzed to remove the traces of ammonia and stored at −20°C. The protein content of whole extract and fractions were determined using the Bradford reagent (Bio-Rad™ Protein Estimation Kit, USA).

Skin prick tests (SPT) and sera collection

Skin prick tests were carried out following Stytis et al. (1982) with crude *L. species* and *S.campanulata* pollen extracts (1:50 w/v). The extract was tested on 182 and 166 adult patients respectively between the age group of 15–55 years with a case history of allergic rhinitis or mild intermittent allergic asthma attending the Mediland Diagnostics Institute, West Bengal for treatment. In addition to crude extracts, SPTs were also done separately with fractions (Fr.30, Fr.60 and Fr.90) of both pollen types, in case of more sensitive patients (Specific IgE, P/N > 3). The detailed history including age, sex, family history, onset and duration of symptoms were recorded. Clinical history and their demographical details of the patients were surveyed through a standardized questionnaire. The patients were also tested for other common allergens like house dust mite, other pollen grains, fungi and food.

SPT was performed with 20 µl of pollen antigen solution placed on the ventral side of the forearm and pricked with a 26 G disposable hypodermic needle. Histamine di-phosphate (1 mg/ml) and PBS were used as positive and negative controls respectively. The reaction was graded from +1 to +4 levels according to Stytis et al. (1982). Corticosteroids and antihistamines were prohibited for 48 hrs before SPT, for the avoidance of decreasing sensitivity of SPT as these anti-allergic medications can inhibit allergic reactions in the skin (Dreborg and Frew 1993). Control sera were collected from two non-sensitized healthy volunteers (confirmed by negative skin reaction) having no history of any previous or current allergic symptoms. Perennial or severe asthma patients, malignancy or other

severe systemic diseases patients, women having pregnancy or lactation and smokers were excluded during skin testing or sera collection. The consents of all patients were obtained before SPT and sera collection. The study was approved by the Ethics Committee of the Mediland Diagnostics Institute, and written consents were also obtained from the patients before SPT and sera collection.

IgE-specific enzyme-linked immunosorbent assay (IgE-ELISA)

Qualitative and quantitative estimation of specific IgE in patients' sera (+1 to +4 against crude pollen extracts) were performed by indirect ELISA (Engvall and Pearlman 1971). Wells of multi sorbant ELISA plates (Nunc, Thermo) were coated with 100 ng/µl crude antigenic extracts (50 µl/well) of pollen and were incubated overnight at 4°C. The wells were then washed with 100 µl of 0.1 M PBS (pH 7.4) plus 0.5% Tween20 (PBS-T) for three times (15 min each) followed by blocking with 50 µl of 1% Bovine Serum Albumin (Sigma) dissolved in PBS-T for 3 hrs at 4°C. After washing with PBS-T for another three times, wells were incubated with 50 µl of pollen-specific patients' sera diluted (1:10) with blocking solution (PBS-T-BSA) at 37°C for 16 hrs. Further washing with PBS-T was followed by incubation with 50 µl of 'Monoclonal Anti-human IgE Clone GE-1-alkaline phosphatase conjugate' (Sigma Chemical Co., USA) in 1:1000 dilution with blocking solution at 37°C for 3 hrs. After final washing with PBS-T, 50 µl of para-nitro phenyl phosphate (pNPP) (Sigma Chemical, USA) liquid substrate was added in each well and incubated at room temperature strictly for 25–30 min in the dark. After the development of color, the reaction was stopped by adding 3N NaOH and OD was taken at 405 nm in an ELISA reader (Multiscan-lab system, Finland). The average OD_{405} value of the replica for each patient's serum was calculated as 'P' and mean of OD_{405} values of all the healthy non-atopic patients was designated an 'N'. For a particular serum, P/N ratio greater than 3.5 was considered as *in vitro* 'positive' with the markedly elevated level of specific IgE (Sircar et al. 2012).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

12% SDS-PAGE was performed for both crude pollen extract and specific AS fraction using discontinuous buffer system according to Laemmli (1970). 20 µl of a sample containing antigenic protein was heated with an equal amount of SDS loading buffer (0.5 M Tris–HCl, pH-6.8, 2% SDS, 5% glycerol, 5% ß-mercaptoethanol, 0.1% bromophenol blue dye) at 100° C for 5 min and loaded in the well of the gel. Electrophoresis was performed using Laemmli buffer system [0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4] at room temperature at 70V (mini gel- 8 × 7 cm) for 2 h 30 min. The protein components were observed by CBB-R250 staining. The gel was documented using Gel Doc 1000 (Bio-Rad, USA) using the MOLECULAR ANALYST software (Bio-Rad, California, USA). The molecular mass of the protein bands was calculated by calibrating with standard marker proteins (Sigma-Aldrich, USA).

IgE-specific immunoblotting

The proteins separated on SDS-PAGE were electrophoretically transferred to PVDF membrane (Sigma Chemicals, USA) by the method outlined by Towbin et al. (1979) using GeNei Mini-Tank blot apparatus at 4°C. The Immunoblotting was performed onto PVDF membrane according to Green and Sambrook (2012). After the transfer, one strip of the membrane was stained with CBB-R250 to ensure the complete transfer of the protein bands onto the PVDF membrane. After transferring, the membrane was cut into 0.5 mm strips and was blocked with 3% BSA in Tris-buffered saline-Tween (TBST, 1 M Tris–HCl, 1 M NaCl, 25 mM $MgCl_2$, pH 7.5) containing 0.05% (v/v) Tween 20 (Sigma Inc, St Louis, USA) for 3 h at 4°C for blocking the free binding sites followed by washing with TBS-T three times for 15 min each. For detection of IgE reactive proteins, the membrane strips were separately incubated overnight (16 hrs) at 4°C with serum (primary antibody) samples in 1:10 dilution, collected from individual patients showing positive skin reaction. Membrane strips were then washed in TBST and incubated with secondary

antibody [Alkaline phosphatase-conjugated monoclonal anti-human IgE (1:1,000), Sigma Chemical Co., St Louis, USA] for 3 h. Binding patterns were visualized by using substrate solution containing nitro blue tetrazolium chloride (NBT) and 5-bromo, 4-chloro, 3-indolyl phosphate potassium salt (BCIP) in 0.1 M TBS, pH 9.5 and the reaction was stopped by 0.5M EDTA.

Results

Aerobiological survey

The two-year aerobiological monitoring in Santiniketan was initiated from January 2013 and continued till December 2014. Here in India, April to early June is considered to be the summer, late June to early September is the monsoon and late September to early November is autumn followed by winter (late November to January) and spring (February to March). Among the airborne pollen grains, *L. speciosa* pollen comprised of 10.5–12% of the total aeropollen load and was found to be prevalent from the end of February to October, with a peak between April and May during summer and then again in July to September i.e. in monsoon (Figure 1(a)). While *S. campanulata* pollen grains were present during spring to monsoon period only (February to August) which contributed 9% of the total aeropollen load with a peak in April (Figure 1(b)). So, *L. speciosa* pollen has two peak pollen seasons; one in summer and one in monsoon while *S. campanulata* pollen has only one peak in the onset of summer. However, their co-occurrence was recorded from February to May and July.

The identification of air borne pollen was done mainly with the help of prepared reference slides by our laboratory and also by consulting published literatures (Huang 1972; Bhattacharya et al. 2006).

Correlation of aeropollen with meteorological factors

Meteorological factors like temperature, rainfall, relative humidity, wind speed were found to be responsible for fluctuations in aeropollen concentration (Andersen 1980; Bricchi et al. 1992; Galan et al. 2000). The correlation matrices for both the pollen grains (*L. speciosa* and *S. campanulata*) have been given in Tables 1 and 2, respectively. From the correlation matrix, it is found that both the pollen grains were positively correlated with temperature, because moderately high temperature with low relative humidity accelerated the pollen dispersal, while they showed a negative correlation with the rainfall, as it adversely affects the movement of pollen grains in the air (Ghosal et al. 2015).

The statistically significant correlation coefficient ('r') values of *L. speciosa* pollen with maximum temperature was 0.46, while it was 0.45 with minimum temperature, with given probability values $p < 0.001$ for all the mentioned variables (Table 1). The wind speed was not found to be statistically significant for *L. speciosa* pollen, while it was significant and positively correlated with '*r*' value (0.54) in *S. campanulata* pollen (p-value <0.001). Both *L. speciosa* and *S. campanulata* pollen showed a positive correlation between the maximum and minimum temperature. Moreover, our results confirmed that rainfall is negatively correlated with both the pollen count ('*r*' value = −0.20 and −0.37, respectively) with a p-value of < 0.001. Thus fewer pollen grains were trapped during heavy rainfall, indicating washing out of pollen grains from the atmosphere. The present findings corroborate with the reports of Hjelmroos (1997) and Ghosal et al. (2015).

Clinical study, SPT, and ELISA results

A total of 1490 surveyed patients [mean age 35 (age range 15–55), Male/Female = 812/678] showed symptoms of cough (89.16%), breathlessness (85.20 %), wheezing (71.5%), sneezing (65.02%), nasal blockage (56%) and skin rash (33.7%) (Table 3). Nasal blocking (86.15%) was the most common symptom among the patients having a history of pollen allergy, followed by sneezing (75.7%) and wheezing (73.6%) (Table 3).

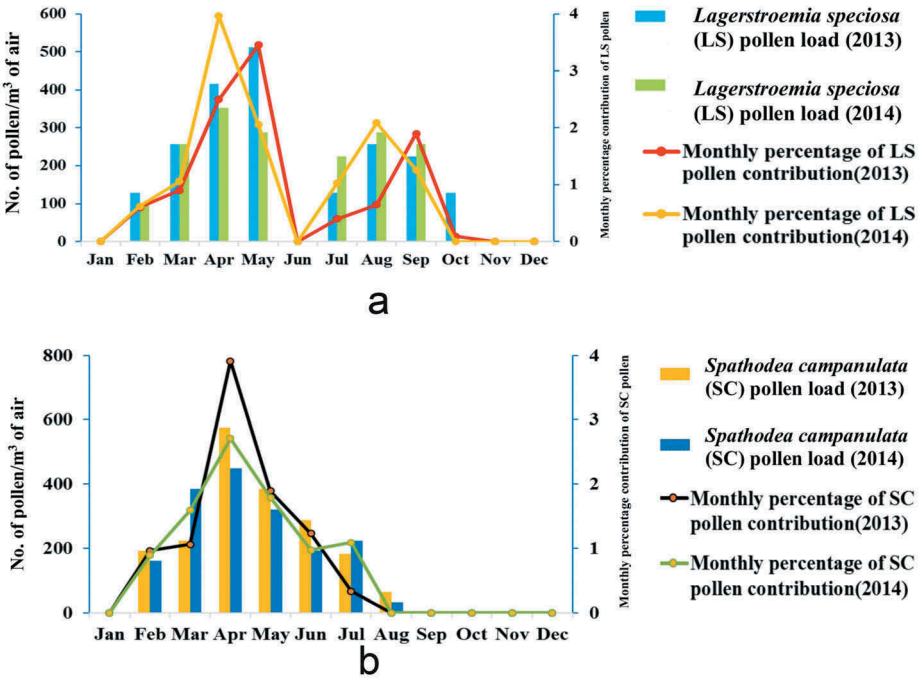

Figure 1. Seasonal periodicity of airborne pollen of *L. speciosa* (a). and *S. campanulata* (b): Bar diagram represents monthly pollen concentration per cubic meter of air trapped by Burkard sampler and line diagram represents the monthly percentage of pollen count for two consecutive years (2013 and 2014).

Table 1. Correlation matrix with p-values for *L. speciosa* (LS) pollen with different meteorological variables.

	Pollen	Max. temp	Min. temp.	RH	Rain fall	Wind speed
Pollen	1.00	0.46(<0.001)**	0.45(<0.001)*	0.34(0.87)	−0.20(0.34)	0.15(0.36)
Max. temp		1.00	0.86(<0.001)	0.68(<0.001)	0.41(<0.001)	0.23(0.09)
Min. temp.			1.00	0.80(<0.001)	0.44(<0.001)	0.07(0.57)
RH				1.00	0.50(<0.001)	−0.19(0.23)
Rain fall					1.00	0.11(0.57)
Wind speed						1.00

Level of significance *0.05, **0.01.

Table 2. Correlation matrix with p-values for S. *campanulata* (SC) pollen with different meteorological variables.

	Pollen	Max. temp	Min. temp.	RH	Rain fall	Wind speed
Pollen	1.00	0.45(<0.001)**	0.41(<0.001)*	0.07(0.87)	−0.37(<0.001)*	0.54(<0.001)*
Max. temp		1.00	0.92(<0.001)	0.70(<0.001)	0.49(<0.001)	0.30(0.01)
Min. temp.			1.00	0.82(<0.001)	0.51(<0.001)	0.10(0.87)
RH				1.00	0.60(<0.001)	−0.24(0.08)
Rain fall					1.00	−0.22(0.10)
Wind speed						1.00

Level of significance *0.05, **0.01.

Skin prick tests were performed with *L. speciosa* and *S. campanulata* crude pollen extracts (1:50 w/v) on 182 and 166 adult subjects, respectively. *L. speciosa* pollen antigen displayed maximum positive skin sensitivity (26.37%) in comparison to *S. campanulata* pollen antigen (22.44%), of which 15.37% and 13.49% patients showed +2 or more sensitizing reactions against *L. Speciosa* and *S. campanulata* pollen antigen respectively (Table 4). By the intensity of skin reactivity in SPT and related symptomization, 23 each of *L. speciosa* and *S. campanulata*

Table 3. Occurrence of different allergic symptoms of respiratory allergic patients visited the allergy clinic (Total patients surveyed and patients suffering from pollen allergy).

Symptoms	Total patients surveyed (n = 1490) (%)	Patients with history of pollen allergy (n = 868) (%)
Cough	1328 (89.16)	486 (56)
Breathlessness	1269 (85.20)	273 (31.5)
Wheezing	1065 (71.5)	639 (73.6)
Sneezing	969 (65.02)	657 (75.7)
Nasal Blocking	834 (56)	748 (86.15)
Skin Rash	502 (33.7)	226 (26)

Table 4. Result of skin prick tests on patients with respiratory allergy using pollen antigenic extracts of avenue trees.

Pollen Allergen Extract	Patients tested	No. and percentage of sensitized patients (+1 to +4) (%)	No. and percentage of sensitized patients showing response ≥+2 (%)
L. speciosa	182	48 (26.37)	28 (15.37)
S. campanulata	166	37 (22.44)	23 (13.49)

polysensitized patients along with two control subjects were selected, and their sera were collected for performing *in vitro* immunochemical test (indirect ELISA). A total of 11 *L. speciosa* sensitive patients and nine *S. campanulata* sensitive patients showed high-specific IgE level (P/N ≥ 3.5) and skin reactivity with various symptoms. It was observed that a cough, nasal blocking and sneezing were the most common and prevalent symptoms among the atopic respiratory patients for both the pollen antigens (Supplementary Table S1 and S2).

Besides the conventional SPTs with crude pollen extracts, SPTs were also carried out separately on the same number of sensitized patients with ammonium-sulfate fractions (Fr.30, Fr.60 and Fr.90) of both the pollen extracts. Among these, the Fr. 60 showed greater sensitization (+3) in *L. speciosa* antigen and Fr. 90 in *S. campanulata* antigen. From the above observations, we could assume that most of the allergic proteins were present in Fr.60 of *L. speciosa* antigen and Fr.90 of *S. campanulata* antigen (Figure 2).

Regarding the reactivity of individual pollen types, it was observed that the patients inhabiting in the area or the surrounding where these two plants grow, showed significantly positive sensitization to the antigens of *L. speciosa* and *S. campanulata*. Aerobiological investigation of monthly variation of these selected pollen types and survey of the allergy-related clinical visit of atopic patients also indicated the significant positive correlation between the seasonal occurrence of these two pollen grains and number of pollen sensitive, atopic patients of the locality (Figure 3). Pearson correlation coefficients between the monthly clinical visit of *L. speciosa* sensitive allergic patients and its atmospheric pollen load was 0.542320 (r^2) (Table 5) indicating a highly significant positive correlation between hospitalization and aerial pollen count. In the case of *S. campanulata* pollen, there was some non-synchronization of atmospheric pollen load, and some sensitized patients visited clinic (Figure 3) which may be due to late sensitization to this pollen. The present result on high incidence of allergy among semi-urban people corroborates with the finding of Wong et al. (2004). Likewise, the number of *S. campanulata* sensitive patients showed significant positive correlation (r^2 value 0.915732 with p-value <0.001) with monthly pollen load in the atmosphere (Table 5). The patients who reported to the clinic with allergic complaints in the respective blooming periods were found to respond significantly to the respective pollen types. A similar correlation between flowering season of plants and their allergenicity were reported for chestnut pollen (Klabuschnig et al. 1981) and also for other pollen types (Erbas et al. 2007). We have formulated two possible regression equation for predicting *L. speciosa* and *S. campanulata* sensitive allergic patients, by which we tried to assume the onset and magnitude of allergic problems caused by the pollen of these two tropical avenue trees (Tables 6 and 7; Regression equation 1 and 2). Allergic symptoms may be seasonal, perennial or irregular depending upon exposure to various allergens. Therefore, it is essential to recognize the nature of symptoms and its

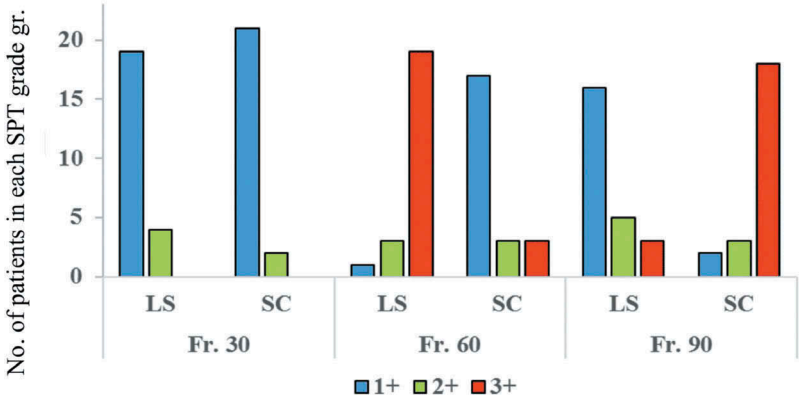

Figure 2. The clustered column chart represents the number of patients across the SPT grade group: The highest SPT grade of sensitization (+3) was shown by Fr. 60 of *L. speciosa* (LS) pollen where Fr. 90 of *S. campanulata* (SC) pollen showed the highest SPT grade of sensitization (+3) in terms of frequency of patients.

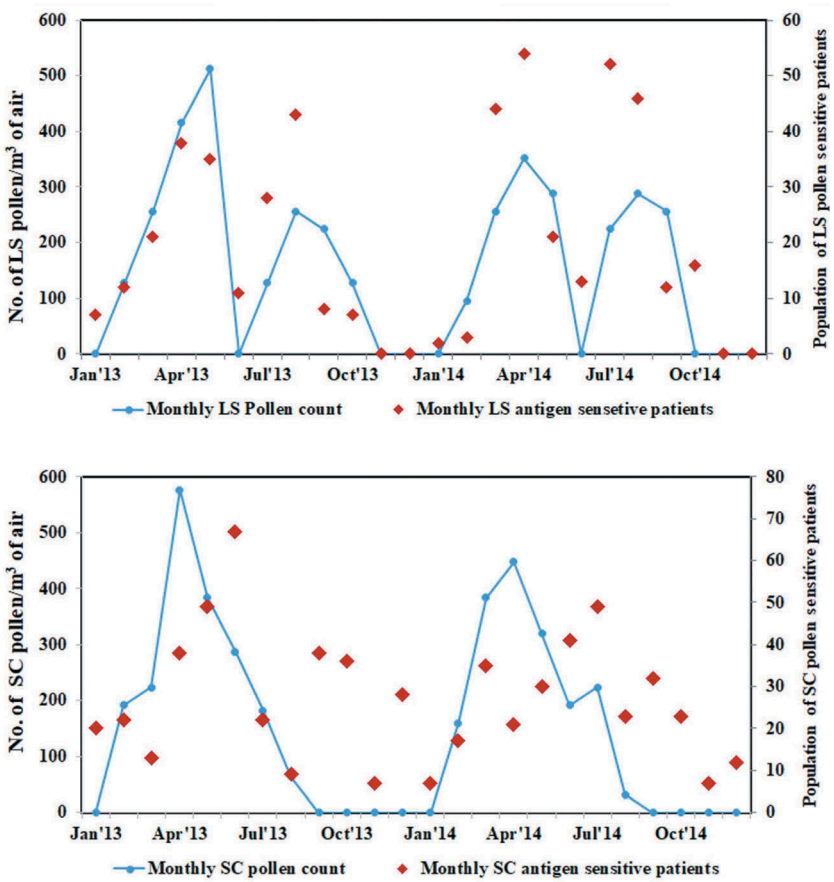

Figure 3. Seasonal pollen load of *L. speciosa* (LS) and *S. campanulata* (SC) vs hospital visit of LS and SC pollen sensitive patients over a period of 24 months: [Population of pollen sensitive patients is represented as scattered plot (red box) along x-axis and monthly pollen count is represented as line diagram (blue line) along z axis].

Table 5. Correlation with *p*-values for monthly clinical visit of LS/SC antigen sensitive allergic patients with monthly LS and SC pollen concentration in the ai

	Independent var.	Correlation value (r^2)	*p*-Value
Number of allergic patients (LS/SC antigen sensitive patients)	LS Pollen count	**0.542320 (Level of significance 0.05)**	<0.0001
	SC pollen count	**0.915732 (Level of significance 0.05)**	<0.0001

Table 6. Multiple regression analysis: LS pollen sensitive patients versus LS pollen count.

Term	Coefficient	Standard error	95% Confidence Interval	*p*-Value
Constant	5.522	3.776	(−2.310, 13.353)	0.1578
LS Pollen count	0.11237	0.02201	(0.06673, 0.15801)	<0.0001

Regression Equation 1:
No. of predicted LS pollen sensitive patients = 5.522 + 0.11237* LS Pollen count in air.

Table 7. Multiple regression analysis: SC pollen sensitive patients versus SC pollen count.

Term	Coefficient	Standard error	95% Confidence Interval	*p*-Value
Constant	4.110	1.713	(0.558, 7.662)	0.0253
SC pollen count	0.143434	0.009277	(0.124195, 0.162672)	<0.0001

Regression Equation 2:
No. of predicted SC pollen sensitive patients = 4.110 + 0.143434* SC pollen count in air.

time of onsets. Case history of patients may also give information if there any relationship exists with patients' allergy symptoms and environmental and genetic factors (Riera et al. 2002). In various studies, similar kind of observation was reported where a higher number of patients attending hospital correlated with higher pollen count in the atmosphere in a specific period (Ordaz et al. 1998; Behbehani et al. 2004; Tobías et al. 2004; Ghosh et al. 2012; Chakraborty et al. 2016).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

On reducing SDS–PAGE (12%), the crude extract of *L. species* pollen antigens resolved into 15 protein bands between 17.2 kDa and 95 kDa with major bands of 95, 64, 60.4, 57, 52, 48, 44, 39.3, 32, 28.2, 21.5 and 17.2 kDa (Figure 4(a)). In fraction Fr.60 of *L. speciosa*, protein components of 95, 64, 57, 52, 48, 44, 39.3, 32, 21.5 kDa were more prominent. The crude extract of *S. campanulata* pollen antigens resolved into 14 distinct bands ranging from 18 kDa and 95.2 kDa (Figure 4(b)), among them 95.2, 81.2, 63.5, 55, 46, 41.6, 37, 33, 27, 24 and 18 kDa were major bands, while 95.2, 81.2, 63.5, 41.6, 46, 33, 27, 24 and 22 kDa bands were most prominent in fraction Fr.90 when stained in CBB.

IgE-specific immunoblotting

Though crude protein antigenic extracts of both *L. speciosa* and *S. campanulata* pollen resolved in SDS-PAGE revealed 14–15 CBB stained protein bands within MW range from 17.2 kDa to 95 kDa. In *L. speciosa* pollen grains, IgE-specific immunoblotting with individual patient sera revealed 7 IgE-binding bands (95, 64, 60.4, 57, 48, 39.3, 21.5 kDa) (Figure 5). Among them, 3 IgE-reactive components of molecular weight 95, 57 and 39.3 kDa were found to be present in all the tested patients showing 100 % binding, while 2 IgE-reactive components (64 and 21.5 kDa) showed 90% and 80% frequency of sensitization when plotting in the heat map (Figure 7). On the other hand, in *S. campanulata* pollen grains, IgE-specific immunoscreening with individual patient sera revealed 5 IgE-binding bands (81.2, 63.5, 55, 41.6 and 22 kDa) (Figure 6) showing 60–100% binding frequency in the heat map (Figure 8). It could be noted that maximum IgE-binding

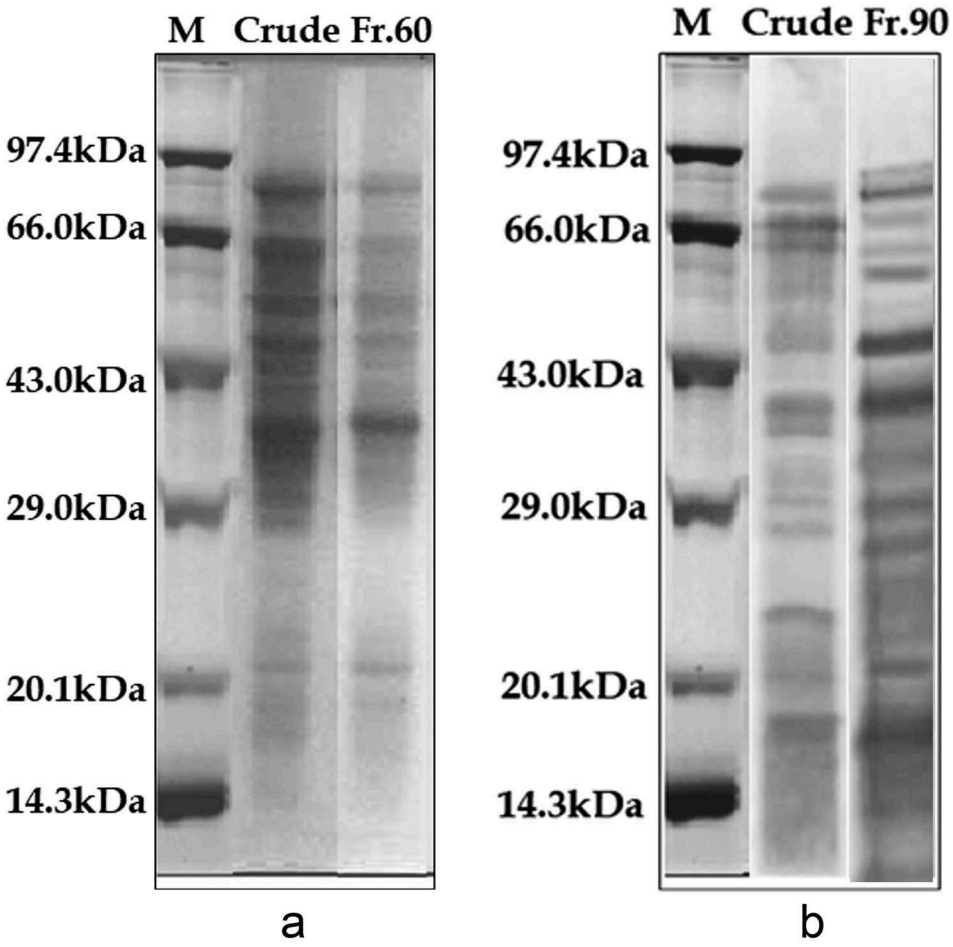

Figure 4. 12% SDS-PAGE of *L. speciosa* crude (20 µl) and Fr.60 pollen extract (20 µl) (a), and 12% SDS-PAGE of *S. campanulata* crude (25 µl) and Fr.90 pollen extract (25 µl) (b) on a 12% discontinuous gel. [M = Molecular Marker, Crude = crude extract, Fr.60 = 60% $(NH_4)_2SO_4$ fraction, Fr.90 = 90% $(NH_4)_2SO_4$ fraction].

proteins of *L. speciosa* antigen were found at high molecular weight range between 39 and 97 kDa, which were present in 60% AS pollen antigen fraction, thus supports our clinical survey.

Among the 5 IgE reactive proteins of *S.campanulata* pollen, four were found to be the most frequent allergenic bands of molecular weight 81.2, 63.5, 41.6 and 22 kDa showing 100% IgE-specific binding with all the tested patients' sera (Figure 8) and most of them were present in Fr.90 which substantiates our clinical *in vivo* investigation. As the major immuno-reactive components were present in Fr.60 and Fr.90 in *L. speciosa* and *S. campanulata* pollen antigen, respectively, these fractions showed more sensitization than the other fractions in SPT (Figure 2). Most of the highly abundant bands visible in SDS PAGE were found to be allergenic to the panel of 19 tested sera sensitive for *L. speciosa* and *S. campanulata* pollen antigen.

Discussion

L. speciosa and *S. campanulata* are two common avenue trees planted along the roadside in Santiniketan of West Bengal state as well as in other parts of India for beautification purpose. According to Basak et al. 2017, *L. speciosa* pollen contributed 2.64% of total yearly total pollen

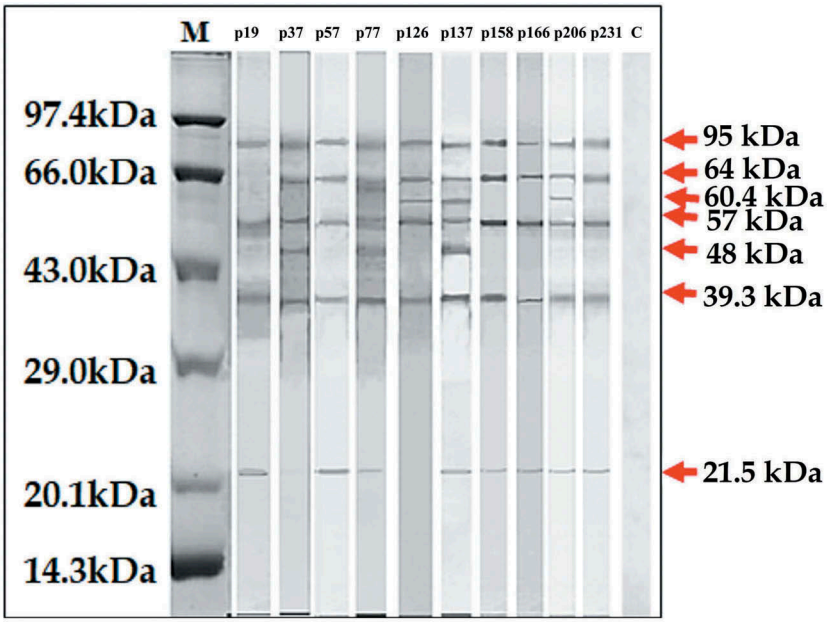

Figure 5. IgE immunoscreening with *L. speciosa* (LS) pollen extract. The lane marked M shows molecular weight markers. Lanes 1 to 10 show immunoblots using serum from individual LS sensitized patients (p19, p37, p57, p77, p126, p137, p158, p166, p206, p231) and Lane C- immunoblot using control serum.

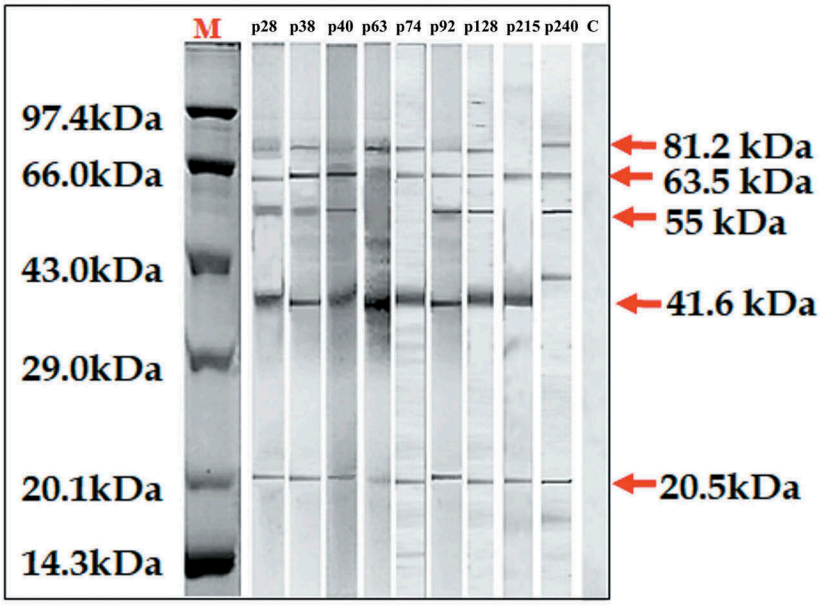

Figure 6. IgE immunoscreening with *S. campanulata* (SC) pollen extract. The lane marked M shows molecular weight markers. Lanes 1 to 9 show immunoblots using serum from individual SC sensitized patients (p28, p38, p40, p63, p74, p92, p128, p125, p240) and Lane C- immunoblot using control serum.

load while pollen of *S. campanulata* contributed 3.02% of total yearly total pollen load which indicated that these pollen types were predominant in the air of the present areas. *L. speciosa* pollens were predominant in the air (10.5–12%) in two distinct seasons, i.e. Summer and

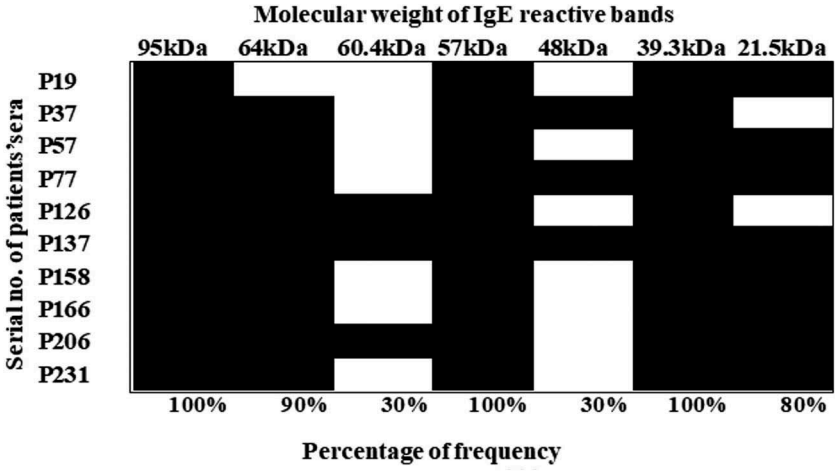

Figure 7. Heat map representing the percentage frequency of sensitization of 7 IgE- immunoreactive components of *L. speciosa* (LS) pollen (black box – present; white box – absent): 95 kDa, 57 kDa and 39.3 kDa bands have shown 100 % IgE specific binding.

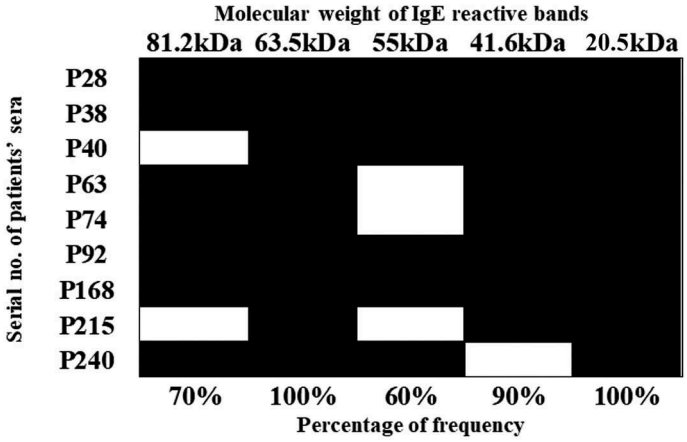

Figure 8. Heat map representing the percentage frequency of sensitization of 5 IgE- immunoreactive components of *S. campanulata* (SC) pollen (black box – present; white box – absent): 63.5 and 22 kDa bands have shown 100% IgE specific binding.

monsoon, while comparatively low concentration (9%) of aeropollen of *S. campanulata* was restricted only to the monsoon season.

As these two are the common avenue trees, people have more chances of being exposed to their pollens. Our study was the first to highlight the importance of *L. speciosa* and *S. campanulata* pollen grains regarding their allergenicity among the Indian population. Till date, there is only one earlier report of their occurrence in the ambient air from West Bengal (Banik and Chanda 1992). The feasibility of using weekly pollen count and weekly meteorological data as dependent and predictive variables were tested by Pearson multivariate nonparametric correlation analysis. Both the pollen types were found to be positively correlated with maximum temperature. Thus our finding supports the view that high temperature promotes an increase in pollen concentration, while a rise in relative air humidity and rainfall cause a decrease in pollen concentration (Ghosal et al. 2015). Pollen of *L. speciosa* and *S. campanulata* showed a negative correlation with the rainfall as they tend to precipitate along with the rain drops (Galan et al. 2000; Jianan et al. 2007). Rainfall and relative humidity showed negative correlations since water droplets washed

away pollen particles (Vega-Murray et al. 2003). The aerobiological investigation also indicated the positive correlation between aeropollen load of these two pollen types and their effect on the allergic reactions amongst the people inhabiting in the localities where these plants grow (Erbas et al. 2007; Osborne et al. 2017). In *L. speciosa*, there was some non-synchronization of atmospheric pollen load and the clinical visit of patients perhaps due to late sensitization to this pollen. A similar relation was also evident about the flowering season of the plants. The patients who reported to the clinic with allergic complaints in the respective blooming periods of these plants were found to respond significantly.

A total of 1490 patients in the age range of 15–55 years with male to female ratio 1.19 [M/F = 812/678] showed cough (89.16%) as a major symptom, followed by the higher prevalence of breathlessness (85.20 %), wheezing (71.5%), sneezing (65.02%), nasal blockage (56%), and skin rash (33.7%). Nasal Blocking is the most common symptom among the patients having a history of pollen allergy. To assess the allergic potency of the pollen antigens tested, only 2+ and higher skin reactions were taken into consideration, since they were likely to be clinically and immunologically significant, unlike 1+ skin reactions, which were often reported as false positive in a certain percentage of normal non-allergic individuals. *L. speciosa* pollen displayed maximum positive skin sensitivity reaction (26.37%), followed by *S.campanulata* pollen (22.44%), of which 15.37% and 13.49% patients induced +2 or more level of reactivity towards antigenic extracts, respectively. In SPT, clinical characteristics of the patients showed co-sensitization with the other allergens viz. 13 tree pollen, three shrub pollen, two grass (Poaceae) pollen, six fungal spores and five foods. The AS fraction (Fr. 60) demonstrated greater sensitization compared to the other fractions (Fr.30 and Fr.90) in *L. speciosa* antigen, while in *S. campanulata* pollen, Fr. 90 was most sensitive in SPT. These were further confirmed by IgE specific immunoblotting analysis. In both the cases, specific pollen sensitive clinic visits of atopic patients were highly significant with respective pollen loads in the air as evident by multiple regression analysis and pollen-specific hospital visit could also be predicted through regression equation. The regression equation was formulated considering the *L. speciosa* and *S. campanulata* pollen load as a predictive variable and the pollen-specific clinical visit of allergic patients as the response variable.

In SDS–PAGE of *L. speciosa* pollen, only five bands (21.5, 39.3, 57, 64 and 95 kDa) were recognized by the 10 tested sera. Among these, IgE-specific protein bands of MW 95 kDa, 57 kDa and 39.3 kDa showed 100% binding frequency in heat map and were considered to be the major IgE-reactive components, while 21.5 kDa and 64 kDa displayed 80–90% frequency of sensitization (Figure 7). All of these IgE reactive proteins were distinctly present in Fr.60 of *L. speciosa* antigen. Thus Fr.60 was found to be the most allergenic fraction which supported our clinical investigation (SPT). In case of *S. campanulata* pollen, SDS-PAGE revealed more than 14 bands, but only two IgE reactive proteins of MW 63.5 and 22 kDa were detected and recognized by all of the nine tested patients' sera. These two IgE reactive proteins are considered as major allergens of *S. campanulata* pollen. Other two IgE reactive bands of MW 41.6 kDa and 63.5 kDa showed 70% and 90% binding frequency, respectively, in heat map which might also be considered as important allergenic components (Figure 8). All four IgE reactive protein components were present in Fr.90 in the SDS-PAGE profile of *S. campanulata* pollen.

Our result provides information on IgE reactive components from *L. speciosa* and *S. campanulata* pollen and widens the scope of further isolation and molecular characterization of the allergens. In the future, purified allergen could be used for the treatment of chronic respiratory disease through immunotherapy.

Conclusion

L. speciosa and *S. campanulata* are two common avenue trees in India. High concentrations of their pollen in the atmosphere, along with high skin reactivity in the atopic patients, suggested that these two plants are important sources of aeroallergens. As these are avenue trees, people have more chance of exposure to their pollen (Manzanoa et al. 2017). Though the atmospheric

presence of their pollen was reported previously, their allergenic reactivity was not tested earlier. It is important to identify the specific pollen season to ensure timely diagnosis of respiratory symptoms associated with pollen allergy. In this connection, we have formulated regression equation predicting *L. speciosa* and *S. campanulata* pollen season which will help in reduction of seasonal allergic manifestation in future that will enable the patients to take preventive measures. Analyses revealed a significant association between pollen counts and some allergy-related hospitalization in West Bengal, India. Our study is the first investigation to analyse allergenicity of *L. speciosa* and *S. campanulata* pollen grains in India, along with detection of the major IgE-binding proteins from these two pollen aeroallergens. These results will provide a good platform for further isolation and molecular characterization of the major allergens from these pollens, which is a prerequisite for the treatment of allergic patients sensitive to pollen grains of these two avenue trees.

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Supplementary Table S1: Clinical characteristics of the 23 LS pollen sensitized patients enrolled in this study.

Patients Sl. No	Age/Sex	Symptoms/ Clinical history*	Grades of SPT**	Specific IgE–titer by ELISA (P/N value)	Co-sensitization with other aeroallergen***
P19	34/M	C,NB,S	+++	3.65	AI,CS,AFu,CD
P22	21/M	W,NB,S	+++	3.27	CN,CS,PE,AAl
P37	20/M	B,NB,S	+++	4.04	CF,CS,PE,AFu,AN
P57	46/M	C,S	++	3.86	Ss,SC,CD,CC,AFu,RH
P61	26/F	W,NB,S	+	1.79	AA,CS,AF
P77	38/F	NB,W,S	+++	3.61	AI,CC,CD,AAl,AFu
P83	55/F	NB,W,S	++	3.09	PS,PE,Ss,CD,AF
P95	28/M	C,NB,Sk	+	1.84	DN,SS,CD,RH
P111	35/M	C,S,W	++	2.74	EC,PP,PE,AAl,RH
P126	33/M	C,S,W	+++	3.86	CP,CF,CS,CC,AFu
P137	41/M	C,S,NB,Sk	+++	4.08	AA,PE,SC,CD,AN,PO
P158	35/F	C,W,S	+++	3.96	SS,Ss,AF,AAl,PO
P166	29/F	C,S,W	+++	3.65	AA,EC,PE,CD,AF
P195	16/M	C,W,Sk	++	2.88	BF,CP,CS,Ss,CD
P199	37/F	C,NB	++	2.78	CS,SC,PP,AF
P202	51/M	W,NB,S	++	3.51	AI,PP,SS,CD,Ss,AN
P206	20/F	S,NB	+++	3.87	PP,Ss,CD,AAl,RN
P211	21/M	C,NB	++	2.97	BF,CP,PE,CS,AN
P214	50/M	B,NB,Sk	++	2.81	SC,CN,CC,AFu,RH
P223	54/F	S,W,NB	+++	3.63	AC,PE,CD,Ss,AN,RH
P224	29/F	S,W,NB	+++	4.07	AA,CS,CC,Ss
P231	36/F	B,W,S	++	3.94	AI,CF,DN,CS,AF,AN
P234	34/F	C,S	++	2.89	Ss,CD,AF,RH
Control patients					
P6	28/M	C,Sk	-	0.68	AC, BF, CN,DN,PP,AFu
P9	41/M	C,B	-	0.72	AI,CN,SS,Ss

P/N: ratio of optical density of patient serum with respect to control

*Symptoms: Cough(C), Breathlessness (B), Whezzing(W), Sneezing(S), Nasal Blocking(NB) and Skin Rash(Sk)

**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 pseudopodes, 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*, DN-*Delonix regia*, EC- *Eucalyptus citriodora*, PP-*Peltophorum pterocarpum*, PS-*Phoenix sylvestris*, PE-*Phyllanthus emblica*, CS-*Cassia sophera*, CC-*Cycas circinalis*, SS-*Solanum symbriifolium*, Ss-*Saccharum spontaenum*, CD-*Cynodon dactylon*, AAl-*Alternaria alternata*, AF-*Aspergillus flavus*, AFu-*Aspergillus fumigatus*, AN-*Aspergillus niger*, RN-*Rhizopus nigricans*, PO-*Pinicillium oxalicum*

Supplementary Table S 2: Clinical characteristics of the 23 SC pollen sensitized patients enrolled in this study.

Patients Sl. No	Age/Sex	Symptoms/ Clinical history*	Grades of SPT**	Specific IgE–titer by ELISA (P/N value)	Co-sensitization with other aeroallergen***
P8	39/M	NB,W,S	+++	2.71	CS,AF,AFu,Ss
P28	21/M	C,NB,Sk	+++	3.66	AI,CC,CD,AAl,AFu
P34	25/M	C,NB,W	+++	2.98	CF,CS,PE,AFu,AN
P38	42/M	B,S,W	+++	3.98	PP,Ss,CD,AAl,RH
P40	28/M	C,B,NB,Sk	+++	3.56	AI,CF,DN,EC,CS,AF,RH
P52	18/F	NB,S	++	2.67	BF,CP,CS,CD,PO
P63	51/F	C,NB,S	+++	3.97	PS,PE,Ss,CD,AF
P74	28/M	B,NB	+++	3.63	CF,CS,CD,AFu,RH
P92	35/M	W,NB,S	+++	4.03	EC,PP,PE,AAl,RH
P98	37/M	S,NB,W	+	1.58	AA,DN,SS,CD
P114	15/M	B,NB	++	3.01	CP,CF,SC,PP,CD,AN,PO
P135	35/F	S,NB,Sk	++	2.77	SS,CD,AF,AAl,PO
P145	29/M	S,W,NB	++	2.35	BF,PP,DR,CS,AF
P159	16/F	B,W,NB	+++	2.97	BF,CP,CS,Ss,CD
P168	27/M	C,NB	+++	3.76	CS,SC,PP,AF
P189	41/F	W,NB,S	++	2.33	EC,PE,PP,AAl,PO
P203	19/M	B,NB,S	++	2.36	AI,Ss,CD,AAl,RH
P206	31/M	C,NB,W	++	1.98	AI,PP,SS,CD,Ss,AN
P215	42/F	C,B,NB	+++	3.65	SC,CN,CC,AFu,RH
P222	47/F	W,NB,S	+++	2.43	AC,SS,CD,Ss,AN,PO
P232	39/F	B,NB,S	++	2.87	AA,EC,PE,CD,AF
P240	26/M	C,B,NB	+++	3.63	AI,CF,DN,PE,CS,,AN
P241	34/M	C,NB	++	1.84	CN,CS,PE,AAl
Control patients					
P6	28/M	C,Sk	-	0.68	AC,BF,CN,DN,PP,AFu
P9	41/M	C,B	-	0.72	AI,CN,SS,Ss

P/N: ratio of optical density of patient serum with respect to control

*Symptoms: Cough(C), Breathlessness (B), Whezzing(W), Sneezing(S), Nasal Blocking(NB) and Skin Rash(Sk)

**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 pseudopodes, 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*, DN-*Delonix regia*, EC- *Eucalyptus citriodora*, PP-*Peltophorum pterocarpum*, PS-*Phoenix sylvestris*, PE-*Phyllanthus emblica*, CS-*Cassia sophera*, CC-*Cycas circinalis*, SS-*Solanum symbriifolium*, Ss-*Saccharum spontaenum*, CD-*Cynodon dactylon*, AAl-*Alternaria alternata*, AF-*Aspergillus flavus*, AFu-*Aspergillus fumigatus*, AN-*Aspergillus niger*, RN-*Rhizopus nigricans*, PO-*Pinicillium oxalicum*

Supplementary Table S3: Detailed questionnaire used in the present survey work for evaluation of clinical health of atopic patients.

QUESTIONNAIRE FOR SURVEY OF RESPIRATORY DISORDERS DUE TO POLLEN IN PATIENTS OF DIFFERENT WORK ENVIRONMENTS
(Department of Botany, Visva-Bharati University, Santinikeatan- 731235
Dist. Birbhum, West Bengal, India)

Sl. No. : Date : Place of work ----------

PERSONAL DATA :
1. Name ----------
2. Address ----------
----------Contact No. : ----------
3. Gender: Male / Female
4. Age : _____Years
5. Date of birth :
6. Occupation :
7. Period of stay in the locality : _____Years

HEALTH STATUS :
Nature of symptoms Duration :
Cough : Yes / No _____Years
Breathlessnesss : Yes / No _____Years
Wheezing: Yes / No _____Years
Sneezing : Yes / No _____Years
Nasal Blocking: Yes / No _____Years
Skin disease : Yes / No Type_____
Others: _____ Yes / No

A. COUGH :
1. Nature of cough : Dry cough _____ Cough with phlegm_____ .
3. Period of symptoms : Seasonal ._____ Perennial _____ Irregular_____ .

4. Worst months		J	F	M	A	M	J	J	A	S	O	N	D
5. Time of onset of symptoms	(a.m.)	1	2	3	4	5	6	7	8	9	10	11	12
	(p.m.)	1	2	3	4	5	6	7	8	9	10	11	12

B. BREATHLESSNESS
1. Nature of breathlessness : With wheezing ._ Without wheezing___ . Exertional _____.
2. Period of symptoms : Seasonal ____. Perennial____ Irregular_____ Other_____

3. Worst months		J	F	M	A	M	J	J	A	S	O	N	D
4. Time of onset of symptoms	(a.m.)	1	2	3	4	5	6	7	8	9	10	11	12
	(p.m.)	1	2	3	4	5	6	7	8	9	10	11	12

C. WHEZZING
1. Nature of Whezzing: Itchiness of nose
2. Period of symptoms : Seasonal-------- Perennial……. Irregular ……Other……

4. Worst months		J	F	M	A	M	J	J	A	S	O	N	D
5. Time of onset of symptoms	(a.m.)	1	2	3	4	5	6	7	8	9	10	11	12
	(p.m.)	1	2	3	4	5	6	7	8	9	10	11	12

D. SNEEZING
1. Nature of symptoms: Frequency/intensity of occurrence
2. Period of symptoms : Seasonal…… Perennial……. Irregular……Other….

3. Worst months		J	F	M	A	M	J	J	A	S	O	N	D
4. Time of onset of symptoms	(a.m.)	1	2	3	4	5	6	7	8	9	10	11	12
	(p.m.)	1	2	3	4	5	6	7	8	9	10	11	12

E. NASAL BLOCKING
1. Nature of symptoms: Stuffy nose/ running nose/ Itchiness of nose
2. Period of symptoms : Seasonal…… Perennial……. Irregular……Other….

3. Worst months		J	F	M	A	M	J	J	A	S	O	N	D
4. Time of onset of symptoms	(a.m.)	1	2	3	4	5	6	7	8	9	10	11	12
	(p.m.)	1	2	3	4	5	6	7	8	9	10	11	12

F. SKIN DISEASE
1. Site of occurrence ----------
2. Period of symptoms: Seasonal….. . Perennial….. . Irregular …….other……...

3. Worst months		J	F	M	A	M	J	J	A	S	O	N	D
4. Time of onset of symptoms	(a.m.)	1	2	3	4	5	6	7	8	9	10	11	12
	(p.m.)	1	2	3	4	5	6	7	8	9	10	11	12

G. OTHER ILLNESS
Heart trouble :Yes / No _____Years
Bronchitis :Yes / No _____Years
Pneumonia :Yes / No _____Years
Sugar : Yes / No
Tuberculosis: Yes / No _____Years
Food/drug/smoke/dust allergy: Yes / No _____Years
Name of food/drug causing allergy ……..
Other: Yes / No _____Years

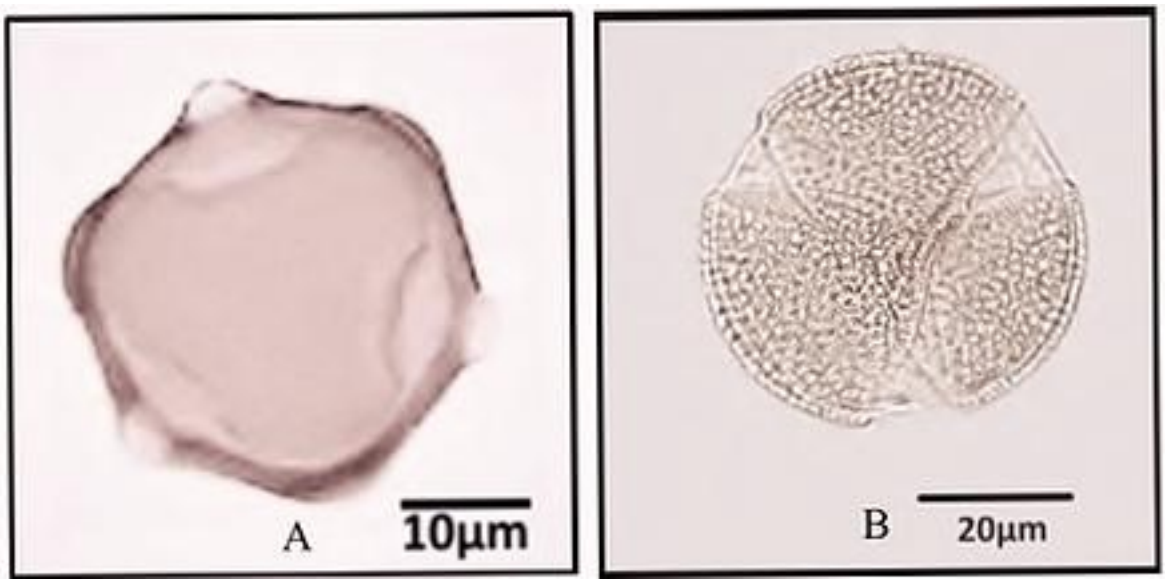
H. SMOKING HABITS
1. Do you smoke? Yes / No
2. If yes Irregularly / Regularly / Chain smoker 3. Type :

I. FAMILY HISTORY
1. Relatives affected : Father / Mother / Grand father / mother . Siblings
2. Brief history in words ----------

J.FOOD allergy:
Name of the fruit/food causing allergy:
_____

Type of symptoms:
Breathlessness/ itching/ asthmatic symptoms/ abdominal pain/ bloating/ vomiting/ diarrhea/ itchy skin/swelling of the skin during hives

Signature of Research Scholar **Signature/ Thump impression of the patient**



Supplentatary Fig.: Pollen microphotographs of *Lagerstroemia speciosa* (A), and *Spathodea campanulata (B)*