



Airborne pollen of *Borassus flabellifer*: Quantification of allergen and antigen

Pampa Chakraborty¹, Sudhiranjan Gupta², Swati Gupta-Bhattacharya¹ & Sunirmal Chanda^{1*}

¹*Division of Palynology and Environmental Biology, Department of Botany, Bose Institute, 93/1 Acharya P.C. Road, Calcutta – 700 009, India;* ²*Department of Botany, Bose Institute, Calcutta, India*
(*author for correspondence: e-mail: sunirmal@boseinst.ernet.in; fax: +91 33 3506790)

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Abstract

Airborne pollen grains of *Borassus flabellifer* were recorded at Madhyamgram, during February to June 1995–1996 commensurate with the plants flowering season. The following year (1996–1997), the exposed Burkard tape segments with the optimal and minimal daily concentrations were divided into two longitudinal equal halves. For each segment, after immunoblotting, one half was incubated with human sera having high IgE titer against *Borassus*, and the other half with anti-*Borassus* rabbit sera for the detection of allergen and antigen respectively. Antigen detection was performed by general immunoblotting method, whereas the allergens were detected by chemiluminescence. The occurrence of the pollen grains in air was compared and correlated with that of the allergen and antigen. The number of allergen and antigen spots were always found to be higher than airborne pollen with great size variation due to the presence of exine free protein particles originating from the pollen grains. The number of allergen spots was always lower than the antigen spots. The occurrence of pollen grains and antigen spots showed stronger positive correlation compared with allergen spots. The peak hours for the occurrence of pollen grains, allergens and antigens were recorded. It is evident from this study that the application of direct aeroallergen monitoring method will be highly useful in allergological research.

1. Introduction

Investigations pertaining to aerobiological survey of pollen in India are being done in different biozones to get information about the daily occurrence of airborne pollen grains including those which are allergenically significant for pollen season forecasting. The essential information for the pollinotics is not the quantity of pollen, but the amount of allergens or at least antigens they bear. Accounts of airborne pollen antigens and allergens have been reported using various immunochemical methods by Agarwal et al. (1981), Schumacher et al. (1988), Takahashi et al. (1993a, 1995a), Rantio-Lehtimäki et al. (1994) and Ekebom et al. (1996). They were mainly concerned with aero-allergen/antigen exposures to ragweed pollen

(Agarwal et al., 1984), Japanese cedar (Takahashi et al., 1993a), grasses (Takahashi et al., 1993b) and birch (Ekebom et al., 1996). This newly emerged approach has been proved to be useful to understand the extent of exposure in pollinotics.

In a country like India with a rich and diverse vegetation, the relevant technique will be very useful to minimize the former tedious and time consuming laboratory procedures. This paper presents a report on aeroallergen and antigen from India, probably for the first time dealing with *Borassus flabellifer* L. (palmyra palm) pollen (Figure 1).

Palmyra palm is a common arecaceous tree which yield sugar, beverages etcetera growing wildy or cultivated in semiarid to arid zones of India, including West Bengal, Bihar and coastal areas of peninsular region.

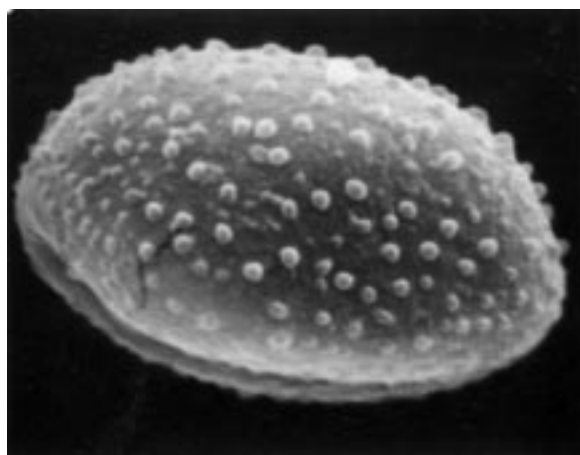
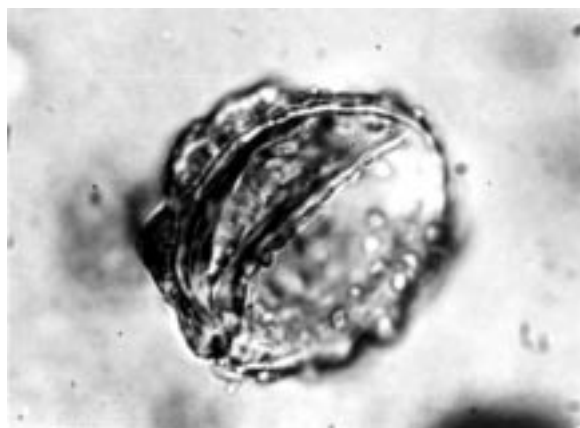


Figure 1. A. Light photomicrograph ($\times 1000$) of the airborne pollen and B. Scanning electron micrograph ($\times 2500$) of the pollen of *Borassus flabellifer* L.

The prevalence of *Borassus* pollen in air and its allergenic significance had already been reported (Banik and Chanda, 1992; Chakraborty et al., 1996).

The objectives of this study are as follows:

1. To record allergens and antigens originating from palmyra palm pollen trapped on the adhesive tapes of Burkard sampler for quantification of actual aeroallergen exposure during the pollen season.
2. To compare and correlate the occurrence of total airborne allergenic pollen with those of its allergens and antigens detected by immunoblotting.
3. To examine the complementary routine airborne pollen counts by direct evaluation of allergens/antigens in air.

2. Materials and methods

2.1. Sampling airborne pollen and selection of exposed tape segments for allergen and antigen immunoblotting

Pollen sampling was performed using a Hirst-type automatic volumetric sampler (Burkard Manufacturing Co. Ltd.) placed on a 0.5 m high concrete base at Madhyamgram Field Station of the Bose Institute, situated in the northern suburb of Greater Calcutta. The presence of *Borassus* pollen in the air was recorded from July, 1995 to June, 1996. The segments presenting each day's record in the month of March when peak concentration was shown in the previous year, were microscopically counted without cover slip. The daily segments of optimal and minimal counts were preserved in sterile petridishes at -20°C until analysis was done.

2.2. Pollen allergen

Freshly collected pollen samples were extracted in phosphate buffered saline (PBS, 0.1M Na-phosphate containing 0.15M NaCl, pH 7.3) by continuous stirring at 4°C for 16 h in 1:50 (w/v) ratio. After centrifugation at $12,500\times g$ for 40 min, the clarified extract was dialysed in PBS and passed through $0.22\text{ }\mu\text{m}$ millipore filter (Millipore Corp., USA).

2.3. Rabbit antisera

Anti-*Borassus* rabbit sera were obtained by immunizing rabbits with whole pollen extract in PBS emulsified with Freund's complete adjuvant (1:1 v/v) mixture (1 ml containing 5 mg protein) applied by intramuscular injections for four consecutive weeks. Intramuscular booster dose was applied similarly with Freund's incomplete adjuvant at four week intervals. After third booster, rabbit sera were collected and stored at -20°C after proper checking by immunodiffusion.

2.4. Skin-prick test (SPT)

Twentyfour *Borassus* sensitive respiratory allergic patients from northern suburb of Calcutta, who had responded previously to *Borassus* allergen, were selected for this study. The pollen types recorded in the Burkard tape segments, considered for immunochemical studies, were used in SPT (Stytis et al., 1982) on the patients with prior consent.

2.5. Human sera

On the basis of SPT report four patients were found to be sensitive exclusively to *Borassus* pollen based on skin reaction. Sera were collected from those patients during the pollen season.

2.6. Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed with four *Borassus* sensitive patient sera with the pollen extracts (100 μ l containing 25 μ g protein/well) recorded in immunochemically treated Burkard segments according to Mitra et al. (1992).

2.7. Aeroallergen/antigen immunoblotting

Each of the 48 mm tape segments were divided into two halves longitudinally for separate allergen and antigen immunoblotting. The transfer was made onto PVDF (polyvinylidene difluoride) blotting membrane (Boehringer Mannheim), which was previously soaked in methanol for 5 min., and then soaked in transfer buffer (25 mM Tris, 38 mM glycine, 20% methanol, pH 8.3). The particle laden segments were then applied to the surface of the membrane, allowing transfer, for one hour at 25 °C. Then the membrane was thoroughly washed in Tris-buffer saline (TBS, 50 mM Tris, 150 mM NaCl, 0.02% NaN₃, pH 7.5) containing 0.02% Tween 20 (TBST) for 3 $\times$ 15 min. and incubated in blocking solution (TBS containing 3% non-fat dry milk) for 3 h at 25 °C followed by incubation of one half with anti-*Borassus* rabbit sera (1:100) and the other with relevant patient sera (1:5) for 16 h at 4 °C. After incubation, the membranes were washed in TBST for 3 $\times$ 15 min. accompanied by shaking. After washing, the membranes were incubated with secondary antibody, [anti-rabbit IgG raised in goat (1:10,000) or anti-human IgE (1:500)] conjugated with horseradish peroxidase (Sigma Chemical Co., USA) in blocking solution for 1 h at 25 °C. Finally, after 3 $\times$ 15 min. washing in TBST, detection of antigen was performed by using diaminobenzidine substrate (Sambrook et al., 1989) and allergen by chemiluminescence following the manufacturer's protocol (Amersham, UK). After removal of excess reagents, in case of allergen detection by chemiluminescence the membranes were covered with Saran Wrap, and an X-ray film (Kodak-X-O'MAT) was carefully exposed in a light safe film cassette. The film was exposed for two minutes and then developed imme-

diately. The allergen and antigen spots were counted under microscope to have a comparative account.

3. Results

The aerobiological survey in the northern suburb of Greater Calcutta from July 1995 to June 1996 showed the prevalence of *Borassus flabellifer* pollen from February to June (Figure 2). The pollen concentration was recorded to be maximum in March, which accounted for 9.71% of the total airborne pollen load.

The following year (July 1996–June 1997), the exposed tapes were examined under microscope during the whole month of March before placing cover slip. The days when *Borassus* pollen showed peak concentration were recorded. During these days the relevant pollen accounted for more than 40% of the total pollen count. Subsequently, a gradual decline in number was recorded. In the days when the allergen and antigen occurrences were recorded by immunoblotting, pollen grains of other taxa were recorded on exposed tape segments as follows.

1. *Areca catechu* (Arecaceae)
2. *Carica papaya* (Caricaceae)
3. *Catharanthus roseus* (Apocynaceae)
4. *Cocos nucifera* (Arecaceae)
5. Cyperaceae
6. *Justicia gendurossa* (Acanthaceae)
7. Poaceae
8. *Trema orientalis* (Ulmaceae)

Twentyfour patients with sensitization to *Borassus* detected by skin test, were further tested by SPT with allergenic extracts of the relevant pollen types along with *Borassus*. *Cyperus rotundus* was selected for Cyperaceae and *Oryza sativa* and *Saccharum officinarum* for Poaceae as the flowering season for these taxa coincided with the study period. After screening on the basis of SPT results, only four patients were found to be exclusively sensitive to *Borassus* pollen. Sera were collected from those patients with prior permission and studied using ELISA technique with the allergenic extracts of the above mentioned pollen types including *Borassus* (Table 1). Three patients out of four showed cross reaction with other Arecaceous members, namely *Areca* and *Cocos*. The remaining one (patient 3) was sensitive to only *Borassus*. After confirmation by both *in vivo* and *in vitro* tests, the serum of patient 3 was used for aeroallergen blotting. The identification of pollen antigen spots was performed by immunoblotting with anti-*Borassus*

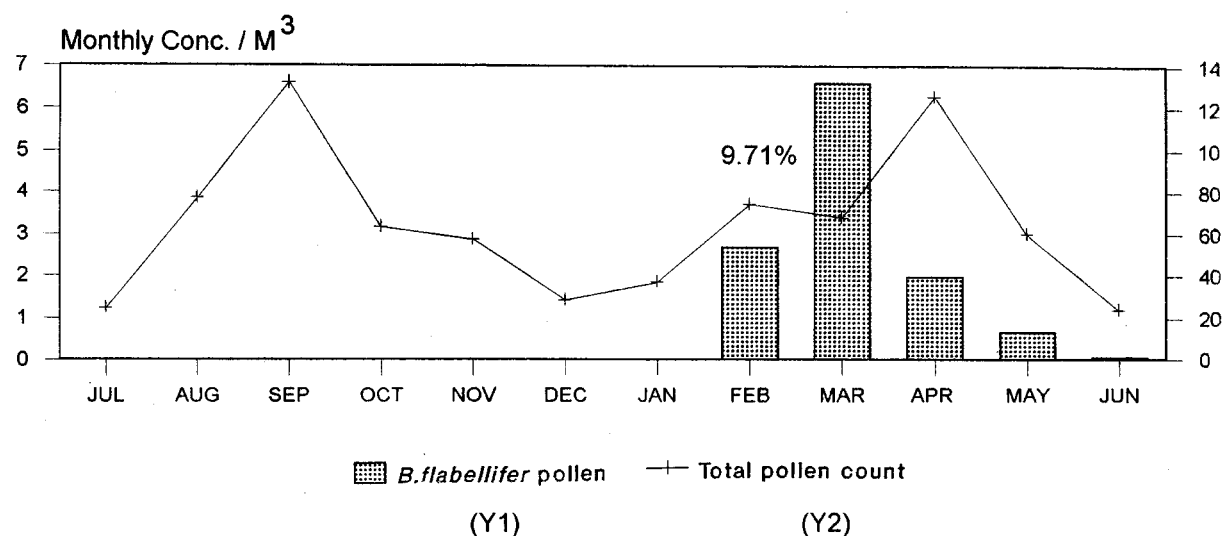


Figure 2. Seasonal variation of the airborne pollen of *B. flabellifer* compared with total airborne pollen load.

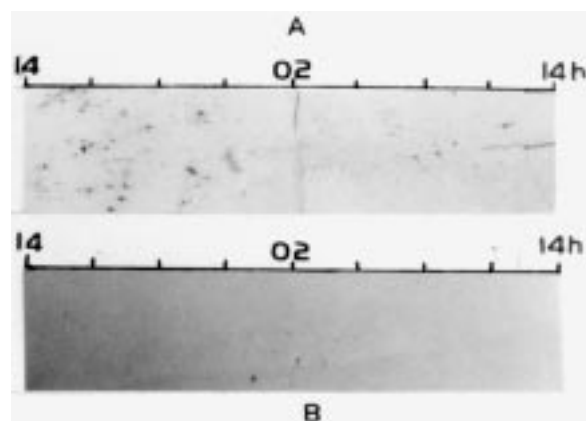


Figure 3. Immunoblotted Burkard tape segments showing allergen spots for (A) March 16/17, 1997 and (B) March 28/29, 1997.

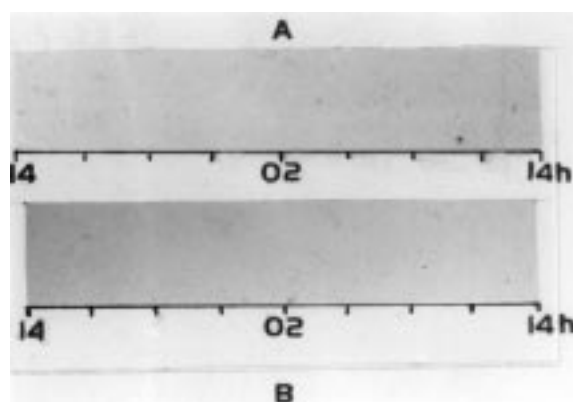


Figure 4. Immunoblotted Burkard tape segments showing antigen spots for (A) March 16/17, 1997 and (B) March 28/29, 1997.

rabbit IgG and recognizable spots were recorded. In case of immunoblotting with pollinotics sera, no detectable IgE spots were found. This was also reported by other workers (Takahashi et al., 1991). Therefore, the detection was performed by using more sensitive chemiluminescent immunoblotting.

After immunoblotting, a number of allergen spots were detected for the exposures of March 16/17 and March 28/29 (Figures 3A and 3B). The result of antigen detection on the other halves of segments of the relevant days is given in Figure 4.

A comparative analysis of the variation of pollen count and allergen spots with time was made for both the days (Figures 5a and 5b). On March 16/17, the occurrence of pollen was recorded throughout the day

with peak count at 18.00 h and 22.00 h. On the same day, the allergen spots showed the peak count at 18.00 h and antigen spots at 17.00 h.

In the case of March 28/29, the peak pollen counts were recorded at 16.00, 02.00 and 03.00 h. Here the allergen and antigen spots were maximum at 03.00 h.

An attempt had been made to observe the correlation (Table 2) between the pollen count and allergen/antigen spots and also between the allergen and antigen spots. The correlation of pollen count and allergen spots as well as antigen spots was positively significant at $> 10\%$ and more than 0.1% level respectively on both March 16/17 and March 28/29. Allergen and antigen spots also demonstrated positive correlation, significant at a level higher than 10% on March

Table 1. Results of ELISA (P/N value)* with different pollen extracts with four patient sera, showing skin-reaction only to *Borassus*.

Pollen allergen extract	Patient 1	Patient 2	Patient 3	Patient 4
1. <i>Areca catechu</i>	2.80	2.40	—	3.60
2. <i>Borassus flabellifer</i>	3.50	3.20	3.40	3.90
3. <i>Carica papaya</i>	—	—	—	—
4. <i>Catharanthus roseus</i>	—	—	—	—
5. <i>Cocos nucifera</i>	—	3.00	—	—
6. <i>Cyperus rotundus</i>	—	—	—	—
7. <i>Justicia gendurossa</i>	—	—	—	—
8. <i>Oryza sativa</i>	—	—	—	—
9. <i>Saccharum officinarum</i>	—	—	—	—
10. <i>Trema orientalis</i>	—	—	—	—

* P/N value is the ratio of ELISA optical density of patient sera and control sera at 492 nm.

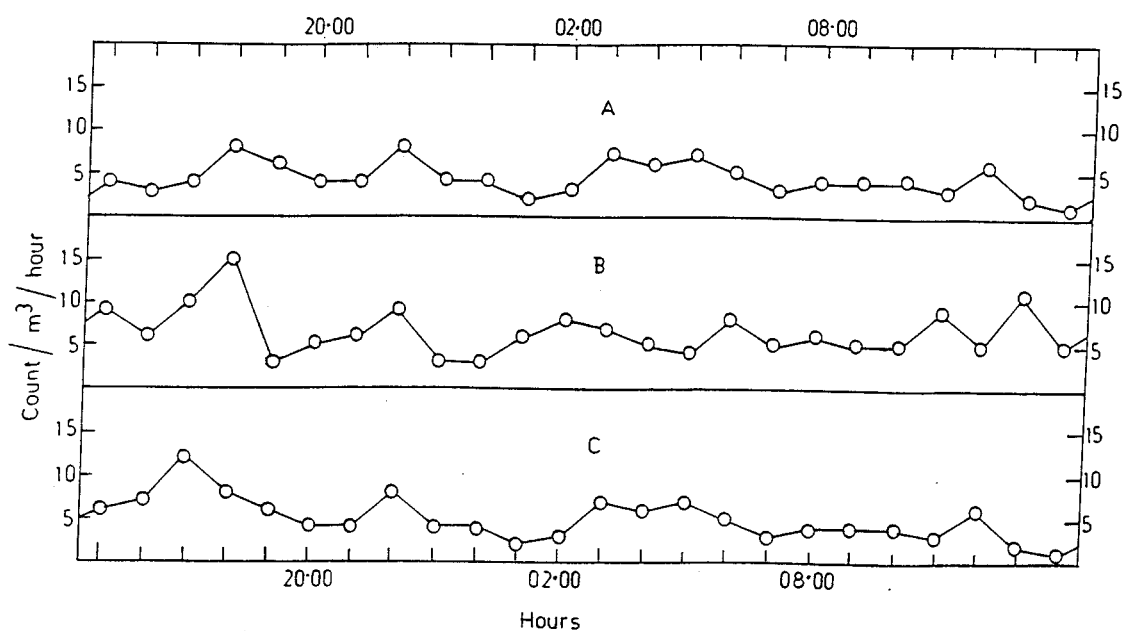


Figure 5a. Hourly variation of (A) Airborne pollen count, (B) Allergen occurrence and (C) Antigen occurrence of *Borassus flabellifer* in March 16/17, 1997.

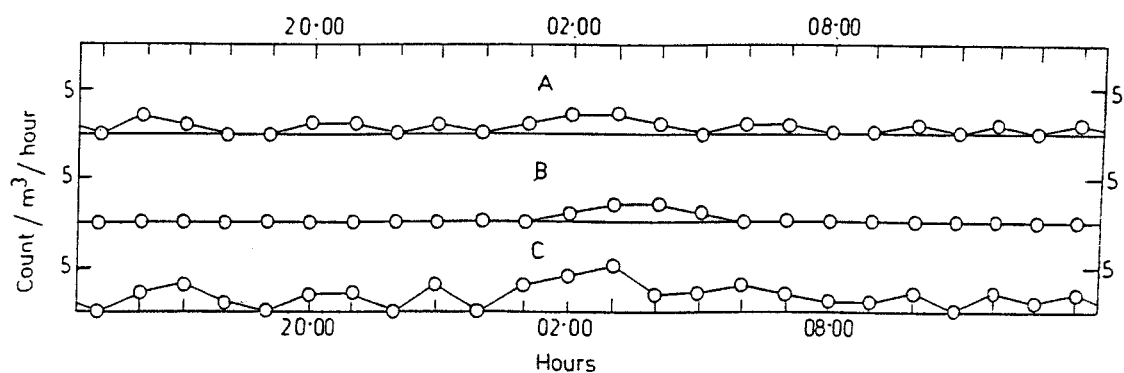


Figure 5b. Hourly variation of (A) Airborne pollen count, (B) Allergen occurrence and (C) Antigen occurrence of *Borassus flabellifer* in March 28/29, 1997.

Table 2. Correlation analysis of airborne pollen count and allergen/antigen occurrence and between allergen and antigen themselves.

	March 16, 1997		March 28, 1997	
	r^*	p	r	p
Pollen grain and allergen spot count	1.78	> 0.1	1.98	> 0.1
Pollen grain and antigen spot count	8.40	> 0.001	7.19	> 0.001
Allergen and antigen spot count	1.78	> 0.1	2.73	> 0.02

* r is correlation co-efficient and p is the level of significance in “ t ” test.

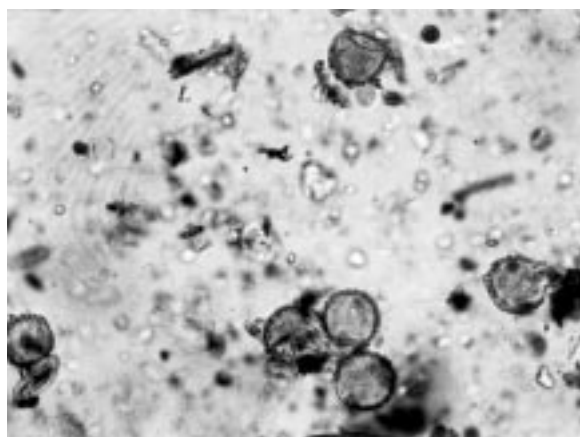


Figure 6. Incidence of airborne *B. flabellifer* pollen on exposed Burkard tape segments under light microscope ($\times 400$).

16/17, and 2% level on March 28/29. However, in all the cases, allergen and antigen spots were always higher in number than pollen counts (Figure 6). Antigenic spots detected by immunoblotting were counted under the microscope, whereas clearly visible allergen spots detected by chemiluminescence were counted directly. Spots were of varying sizes, ranging from 5–150 μm in diameter.

4. Discussion

The results of this investigation demonstrated that the pollen grains of *Borassus flabellifer* are dominant aeroallergens during its flowering period. The airborne pollen grains showed stronger positive correlation ($p > 0.001$) with antigen than with the allergen ($p > 0.1$). Allergen and antigen also, showed positive correlation, but not highly significant ($p > 0.1$) during the peak days, but were significant at a comparatively higher level ($p > 0.02$) in the days of lower concentration. From the results, it can be concluded that the

protein particles having antigenicity were not always allergenically active.

5. Conclusions

The difference between pollen count and number of allergen/antigen spots, and the variation of spot size in both cases, are indicative of the fact that the air transports not only pollen, but also tiny antigenic or allergenic particles. Airborne pollen grains as larger particles (15–50 μm in size) are trapped in the upper respiratory tract and reach bronchi in small amounts. Nevertheless, a fair proportion of pollen allergic patients experience respiratory problem during the pollen season. This is probably because they get exposed to pollen allergenic particles much smaller in size than whole pollen grains. The grains after coming in contact with the moisture, dew or rain-drop, probably release its soluble protein which, in turn, become airborne in the form of tiny particles, inhaled and reach the lower respiratory tract (Mygind et al., 1996). The existence of smaller airborne allergenic pollen particles has already been reported for ragweed, birch, grasses and Japanese cedar pollen (Agarwal et al., 1984; Takahashi et al., 1991, 1995b; Rantio-Lehtimäki et al., 1994; Spieksma et al., 1995). The larger spots ($> 50 \mu\text{m}$, the average diameter of *Borassus pollen*) originated probably as a result of merging of a number of spots and consequent diffusion during the transfer for immunoblotting (Takahashi et al., 1993a).

The study, however, had some limitations of allergen/antigen spot counting with absolute accuracy due to size variation (approximately 5–200 μm or more) and sometimes due to overlapping. Nevertheless, the occurrence of airborne allergen or antigen obtained through such investigations is relatively cheaper and fairly reliable.

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