

Airborne rice pollen and pollen allergen in an agricultural field: aerobiological and immunochemical evidence

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Received 4th March 2003, Accepted 5th September 2003

First published as an Advance Article on the web 6th October 2003

Grass pollens are well known among the health hazardous bioaerosols causing respiratory allergy. Being an important member of the grass family, the rice plants contribute a huge pollen load in agricultural fields during flowering. This results in a seasonal trigger of hay fever and respiratory allergy in the field workers and people living in the vicinity. Studies on the monitoring of airborne rice pollen and the intensity of the released allergen in agricultural fields are largely lacking. The aims of the present study were: (1) daily and hourly monitoring of airborne rice pollen in an agricultural field during the flowering period of plants in a winter crop season by using the Burkard 7-Day Volumetric Sampler and (2) the measurement of hourly airborne allergen intensity in the field in a peak rice pollen period by the double-antibody and chemiluminescence techniques to find out its relationship with the airborne rice pollen concentration. The monthly average concentration of rice pollen was 95 pollen m⁻³ and the range of daily average pollen concentration was 0 to 386 pollen m⁻³. A bimodal diurnal periodicity showed that the airborne rice pollen concentrations remained high at two different times of the day: between 08:00 h to 12:00 h and 14:00 h to 16:00 h. Deposition of airborne rice pollen allergen showed darker intensities on the immunostained tapes from the Burkard Sampler at the same two positions corresponding to higher pollen counts. These observations provided direct evidence of the allergenicity of airborne rice pollen in field conditions.

Aim of investigation

Many pollen grains are potent sources of allergens of which airborne grass pollen is the most significant due to its high prevalence all year round (Ogden and Lewis,¹ Chanda and Nandi,² Smart and Knox³). Grass pollens have been known to trigger respiratory allergic responses for a long time (Ordman,⁴ Suphioglu *et al.*⁵). Beside intact pollen grains, antigenic particles emerging out of the pollen also get released into the air, which in association with other bioaerosols may induce respiratory allergic symptoms (Spieksma *et al.*,⁶ Rantio-Lethimäki *et al.*⁷). Among the different sources of airborne grass pollen, rice held an important position because of its wide cultivation round the world. Asthma episodes corresponding to the flowering season of rice plants (Matsumara *et al.*⁸) and impaired respiratory health among the rice farmers (McCurdy *et al.*⁹) were reported earlier. Information on the airborne rice pollen load in the fields during the flowering periods can help the field workers and the people staying in the vicinity to take precautionary measures in advance. It appears, however, that studies on the monitoring of airborne rice pollen and the intensity of the released allergen in agricultural fields are largely lacking. Although there are reports on the clinical and biochemical studies on grass pollen allergens including one report on rice (Xu *et al.*¹⁰), so far there has been no direct evidence proving the allergenicity of individual grass pollen types while they are airborne in field conditions. The reasons

behind this were the problem of identifying individual airborne grass pollen up to species level, as they are mostly morphologically indistinguishable, and lack of the proper immunochemical techniques. The problem of identification has been solved for rice pollen because airborne rice pollen grains are now identifiable following the recently published identification criteria for Indica rice varieties (Chaturvedi *et al.*¹¹). Following these criteria, rice pollens were separately analyzed in this study, isolating them from the cumulative grass pollens collected in the field. A detection and quantification method for airborne birch pollen allergens on polyvinyl difluoride (PVDF) membranes by using immunoblotting and chemiluminescence was reported by Ekeboom *et al.*¹² Similar methods were reported by Ramzmovski *et al.*¹³ for the airborne pollen of *Lolium perenne*. This method was used in this study to detect the intensity of the allergenic components released from airborne rice pollen grains in the field. The airborne pollen grains originating from a local common Indian variety of rice, namely *Oryza sativa* var. Ratna, were targeted in this study. This rice variety was selected because it is widely cultivated in the West Bengal state of India and a large number of people are occupationally involved in its farming. A Burkard 7-day Volumetric Sampler (Burkard Manufacturing Co. Ltd., Hertfordshire, UK) was installed at the central position of an experimental rice field and daily and hourly monitoring of the airborne rice pollen was performed during the flowering period of the plants. Precisely, the present study was undertaken to determine: (1) the daily and hourly concentration of airborne rice pollen in an agricultural field during the flowering period of the winter crop season and (2) the hourly airborne rice pollen allergen intensity in the field in a peak rice pollen period by using the double-antibody and chemiluminescence techniques to find out the correlation between the diurnal changes of rice pollen concentrations in the air and similar variations in the allergen load.

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Description of experimental procedures

Sampling of airborne rice pollen in the field

A Burkard 7-day Volumetric Sampler was installed on a 0.5-m concrete base (the orifice of the sampler was at a height of about 1 m) in a square plot (20 m × 20 m) of rice (*Oryza sativa* var. Ratna) crop at the Madhyamgram Experimental Farm of the Bose Institute. Air sampling was started when the rice buds became mature, about to bloom, and continued for one month (8th October–6th November) until the end of the flowering period of plants during the winter crop season.

The Burkard 7-day Volumetric Sampler sampled air uninterrupted for a seven-day stretch and the airborne particles were laden on a melinex tape coated with petroleum wax. The exposed tape was then segmented into seven pieces, each being 48 mm long, representing 24 h of exposure. Then the tapes were mounted with gelvatol in a microslide. Slides were scanned by a high-resolution light microscope (Labophot 2, Nikon Corporation, Japan) for counting pollen along the traverses at 2 mm corresponding to one-hour sampling. The counts of pollen grains were converted into the mean hourly concentrations (pollen m⁻³) according to the instructions of the British Aerobiology Federation.¹⁴ The 24 hourly concentrations were then averaged to get the daily mean hourly concentrations, which in turn provided the monthly mean concentration. For the study of diurnal periodicity, those days when the rice pollen concentration exceeded the overall mean concentration were selected. The hourly counts of these ideal days were normalized by dividing by the respective daily mean count and the average data for each hour were then plotted to obtain the diurnal periodicity.

Pollen collection and preparation of pollen extracts for the immunochemical study

The floral panicles of the selected rice variety were dried overnight in an incubator (<40 °C) and then mildly crushed in a blender. After sieving, the pollen grains were collected at almost 95% purity level and preserved at -20 °C for further use.

Samples for skin-prick testing and electrophoresis were prepared by extracting the pollen grains with 95% purity in phosphate buffered saline, i.e., PBS (0.2 M Na-phosphate, 0.15 M NaCl, pH: 7.2) overnight at 4 °C in a magnetic stirrer with occasional stirring. Then the material was centrifuged in an automatic high-speed refrigerated centrifuge (Model CR20B2, Hitachi, Japan). The supernatant was taken and passed through a 0.22 µm Millipore filter (Millipore Corp. Bedford, MA, USA) and stored in different aliquots. One part from each extract was kept for skin-prick testing of the allergy patients and the rest was stored at -20 °C for future use after dialyzing in cellulose membrane of molecular weight cut off 12,000 dalton (Sigma, USA). The protein content in the extract was measured following the method of Lowry *et al.*¹⁵

Methods for skin-prick test and preparation of human serum pool

For skin-prick testing subjects were selected based on their positive clinical history to pollen allergy. The patients attending the Allergy Clinic of Institute of Child Health, Kolkata were subjected to skin-prick tests with the sterile allergen extract of rice pollen following the standard methods (Shivpuri and Agarwal,¹⁶ Wahl *et al.*¹⁷). The allergen extracts were applied on the forearm of the patients and each site was pricked using a sterile hypodermic needle (No. 26). Phosphate buffered saline was taken as '-'ve and histamine diphosphate as '+'ve control. The intensity of reactions with respect to the wheal or erythema responses was measured after 20 minutes. The reactions were classified into following categories (Terr¹⁸): negative reaction (no wheal or erythema), 1+ (no wheal, erythema diameter

<20 mm), 2+ (no wheal, erythema diameter >20 mm), and 3+ (both wheal and erythema).

The sera were collected from the patients with marked positive reactions (>2+). None of the patients were allowed to take medicine for at least 48 h before performing the test. A patient serum pool was prepared from ten individuals with a positive clinical history suffering from rice pollen allergy as confirmed by skin-prick tests. The pooled patient serum sample was then kept in aliquots and stored at -20 °C for further use. The same serum pool was used in all cases.

Immunoblotting of allergens from exposed Burkard tapes

The 48 mm portion of the pollen-laden tape, corresponding to 24 hours (14:00 h to 13:00 h) during the peak rice pollen week, was cut and the allergens were then transferred to the PVDF membrane using the capillary transfer method. The membrane was placed in a washing solution (0.2 M PBS, 0.05% NaN₃) containing 2% BSA (Bovine Serum Albumin) for blocking at 4 °C overnight. After washing, the membrane was transferred to a second washing solution containing human IgE, diluted to 1 : 50. It was incubated for 30 minutes and after rewashing transferred to a third washing solution containing Goat-anti-human IgE conjugated with HRP (horseradish peroxidase) diluted at 1 : 500 ratio and then incubated for 30 minutes (Ekeboom *et al.*¹²). Prior to chemiluminescence treatment, the membrane was washed three times in washing buffer. The membrane was then subjected to chemiluminescence reaction according to the manufacturer's protocol (Amarsham, Freiburg, Germany).

After completion of this process the immunoblotted PVDF membrane was scanned by an image analyzer (BioRad, California, USA) to measure the optical density of each hourly immunoblotted segment. The total membrane area was divided into 24 equal segments and each segment represented one hour equivalent to the exposed Burkard tape.

Results and discussion

The range of daily rice pollen concentration from the one-month sampling during the flowering period of plants was 0–386 pollen m⁻³. The monthly average pollen concentration was 95 pollen m⁻³. The maximum pollen load (>95 pollen m⁻³) was present in the air for eight days, between 21st and 29th October. Daily variation of rice pollen concentration is presented in Fig. 1.

For estimating the diurnal periodicity pattern of airborne rice pollen grains, those eight days when daily average rice pollen concentration exceeded 95 pollen m⁻³ were selected. The hourly pollen counts of those days were normalized by dividing by the respective daily mean counts and the average pollen

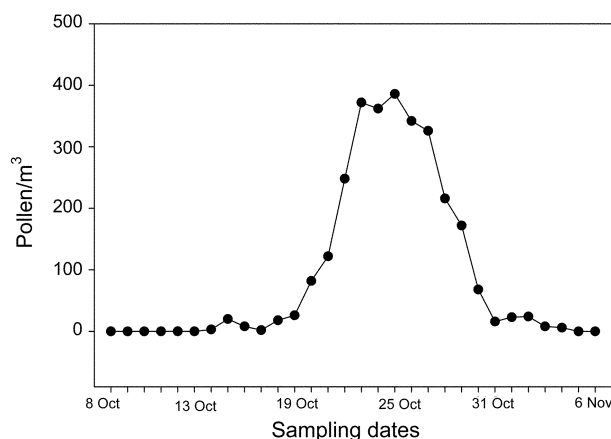


Fig. 1 Daily variation of airborne rice pollen concentrations (pollen m⁻³) during the one-month sampling period.

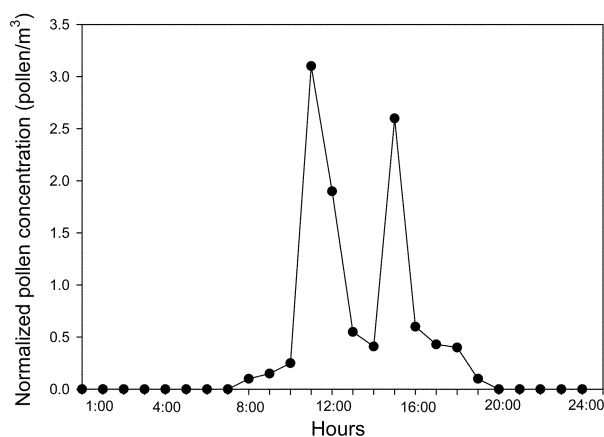


Fig. 2 Diurnal periodicity of normalized hourly mean concentration (pollen m^{-3}) of airborne rice pollen.

concentration data of each hour was then plotted to obtain the diurnal periodicity (Fig. 2). The diurnal periodicity indicates that rice pollens were prevalent in the air during the period from late morning to late afternoon (11:00–16:00 h). From 08:00 h onwards the airborne pollen load was increasing, however, after 11:00 h the concentration started to decrease. The second pollen peak again started from 14:00 h onwards and started to decrease after 16:00 h, resulting in a typical bimodal frequency pattern of diurnal periodicity, which was in agreement with Gregory's¹⁹ observation for airborne grass pollen grains. No rice pollen was recorded in the early morning and night hours. Earlier researchers have reported diurnal variation of airborne grass pollen (Galan *et al.*,²⁰ Norris-Hill and Emberlin,²¹ Spikesma & den Tonkelaar,²² Trigo *et al.*²³), however most of these studies were restricted to urban areas away from the pollen sources. Distance from the pollen sources, pollen release time, and environmental variables affecting pollen dispersal might limit the accuracy of the estimation of diurnal periodicity by the previous researchers. Moreover, the diurnal periodicity data of cumulative grass pollen cannot properly reflect the diurnal periodicity of specific grass pollen, generated in a huge quantity from a widely cultivated crop. As the diurnal periodicity information is important in relation to the protection against work related pollen allergen exposure, further detailed study near the pollen source and better understanding was worth pursuing. The present study is anticipated to fill these knowledge gaps. The diurnal periodicity data of airborne rice pollen achieved from our experiments performed at the center of a rice field is expected to be more significant compared to the previous studies.

To determine the allergenic potential of rice pollen, general allergic subjects with the symptoms of respiratory allergy (hay fever or allergic rhinitis, asthma, sinusitis, *etc.*) were selected first. 240 patients were selected from this group for skin-prick test, who had positive clinical history to pollen allergy and who reported higher symptoms of respiratory allergy during the rice flowering periods. Patients were selected based on a questionnaire of allergy symptoms. However, patients kept no symptom diaries. The skin-prick tests using the rice pollen extract in these 240 allergy patients demonstrated positive reactions in 161 (66%) cases. Positive reactions of skin-prick tests showing grades of sensitivity ranging from 1+ to 3+ are presented in Table 1. Among the rice pollen allergen positive patients, 152 (94%) cases demonstrated 1+ sensitivity reaction, which developed no wheal but erythema of <20 mm diameter; seven cases (4%) demonstrated 2+ reaction, which developed no wheal, but erythema diameter of >20 mm; and only two persons (1%) showed 3+ reaction with both wheal and erythema. While performing skin-prick tests, a cross reactivity

Table 1 Results of skin-prick test on pollen allergen sensitive patients using the antigenic extract of rice pollen

No. of patients tested	Grades of sensitivity			Percentage of positive cases
	1+	2+	3+	
240	152	7	2	66.25%

was observed between the pollen allergens of the different members of the grass family. *Oryza sativa* var. Ratna pollen specific IgE derived from the serum of sensitive patients cross reacted with the pollen allergens of other rice varieties and with a wild grass, *Phragmites karka* (results not shown).

Immunoblotting of exposed Burkard tape of the peak rice flowering period demonstrated that, similar to airborne pollen, the aeroallergen intensity also remained high between 11:00 h to 16:00 h of the day. The variation of optical densities (Fig. 3) indicated that the allergen intensity was greater during the period between 07:00 h to 10:00 h (mean OD range: 0.131–0.132) and between 14:00 to 17:00 h (mean OD range: 0.118 to 0.083). The hourly airborne rice pollen concentration from the microscopic analysis for that particular time (Fig. 4) corroborates this result. A positive correlation ($r = 0.42$) was found between the variation of hourly airborne pollen concentrations and the hourly allergen intensities. While comparing the number of allergen spots on the immunostained tape with the number of pollen counted by the microscope in the corresponding time period, the numbers of allergen spots were found to be higher. This observation indicates that the airborne

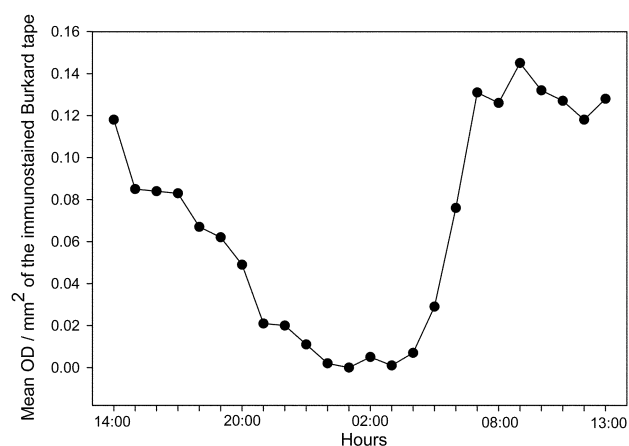


Fig. 3 The diurnal variation of allergen intensities on the rice field in terms of mean optical densities in the immunostained Burkard tape.

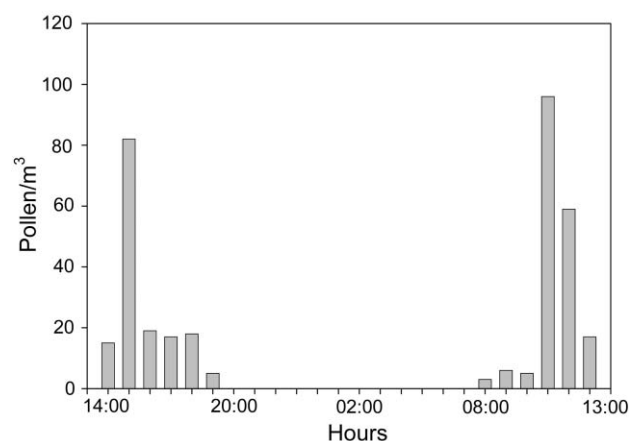


Fig. 4 The hourly airborne rice pollen concentration (pollen m^{-3}) for the same period of time corresponding to the immunostained Burkard tape.

concentrations of allergenic components should be higher than the corresponding intact airborne pollen grains because of the release of allergens through the apertures as well as due to the allergenically significant airborne plant fragments associated with the pollen. It was also observed that the spots were larger in size than the size of pollen grains. Several immunoblotted spots were visible even to the naked eye. This may be due to the result of the diffusion during allergen transfer as was reported by other researchers (Takahasi *et al.*²⁴) or may be due to the aggregation of allergens from a group of pollen grains. These results proved directly the presence of allergenic components above the rice field during its flowering. A similar immunostaining of the Burkard tape was done during the last phase of rice flowering and decreased numbers of allergen spots were observed there (results not shown).

A high concentration of airborne rice pollen during the flowering, positive skin-prick test reactions of the pollen allergen sensitive patients against the antigenic extract of the rice pollen and finally the similar patterns of diurnal periodicities of airborne pollen and pollen allergen indicated that the rice pollen is an offending aeroallergen for the agricultural field workers as well as the people staying in the vicinity of the rice fields. The obtained results are expected to be of use for the clinicians and aerobiologists to forecast the rice pollen dispersal in the air and consequent allergic reactions in susceptible patients. The diurnal periodicity information will be valuable for the farmers to take respiratory protection measures against the work related exposure to airborne allergenic rice pollen in the fields at the time of rice flowering.

Acknowledgements

The authors acknowledge the financial support received from the Ministry of Environment and Forests, Government of India (Sanction No. 42/8/94-RE). The authors are thankful to Dr. Indrani Roy and Dr. Soma Chatterjee of the Institute of Child Health, Kolkata for the help rendered during skin-prick tests. Thanks are due to Dr. Pampa Chakraborty for her critical suggestions during the manuscript preparation and Mr. Chanchal Chakraborty for sampling arrangements during the field study. They also wish to acknowledge the help received from Dr. Alastair McCartney of IACR-Rothamsted, Harpenden, Herts, UK, for his valuable suggestions in making the calculations regarding the Burkard volumetric sampling.

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