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ANTIGENIC AND ALLERGENIC PROPERTIES OF *AMARANTHUS SPINOSUS* POLLEN – A COMMONLY GROWING WEED IN INDIA

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Abstract: *Amaranthus spinosus* (Fam. Amaranthaceae) is an important aeroallergen in India and grows commonly in different parts of the country. In spite of its clinical significance in Type I hypersensitivity disorders, the antigenic and the allergenic properties of the pollen have not been systematically resolved. We investigated antigenic and allergenic properties of 5 pollen samples of *Amaranthus spinosus* collected from the Delhi area at fortnightly intervals. The protein content did not exhibit statistically significant variability. However, samples collected during the peak flowering season showed higher protein content. Biochemical characterization of samples showed multiple protein fractions by IEF and SDS-PAGE analysis. Samples collected during peak season showed a slightly higher number of bands (22) in the mw range of 14–70 kD. Seven protein fractions of 70, 66, 60, 50, 40, 30 and 14 kD were observed to have IgE binding capabilities and 9 were treated as allergenic. The observations will be helpful in standardizing pollen antigens for diagnosis and immunotherapy in India.

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The importance of the *Amaranthus spinosus* pollen in triggering respiratory allergy is well established and documented in India [9, 16, 17, 18]. In spite of its clinical significance in the elicitation of immediate hypersensitivity disorders, the antigenic and allergenic properties of the pollen have not been systematically resolved. It belongs to the family Amaranthaceae and produces an enormous amount of pollen to be disseminated in the atmosphere. Subsequently, we documented a high concentration of its pollen in the atmosphere in Delhi, and other investigators did so in other parts of the country [1, 2, 14, 18, 22]. The information on the antigenic and allergenic components of *A. spinosus* will be helpful in the production of safe, effective and standardized diagnostics and therapeutic allergen extracts in the country. Therefore, the present study is aimed at finding an antigenic and allergenic profile of *Amaranthus spinosus* pollen collected at different time intervals during the same pollination season and from the same locality.

MATERIALS AND METHODS

Collection of pollen. *A. spinosus* is a naturally growing annual weed on vacant and wastelands (Fig. 1). It flowers throughout the year, but the main flowering season is from August–October, after the rains. Five samples of the polliniferous material were collected from the field at an interval of about 2 weeks between July–September from Delhi from the same locality. After separation of extraneous materials, the inflorescence was dried at 37ºC and naturally shed pollen were passed through different grades of sieves to obtain pure pollens. Pollen purity was checked per the Cour and Loublier's method [3]. Pollen samples showing more than 95% purity were included for

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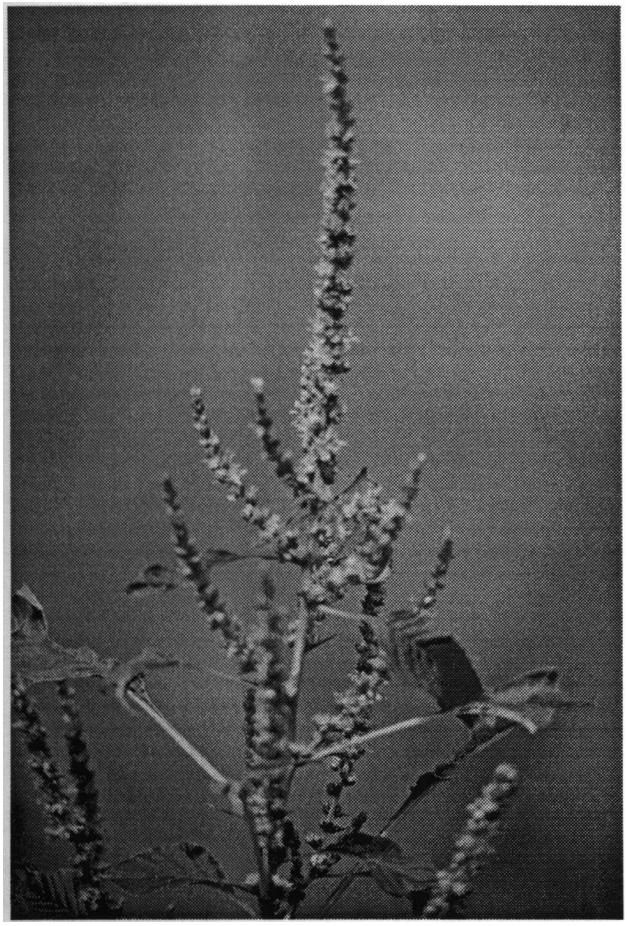

Figure 1. *Amaranthus spinosus* weed growing along the road sides and waste places in Delhi.

further investigations. The pollen of *A. spinosus* are spherical, pantoporate and exine surface scabrate, 22–26 µm in diameter (Fig. 2).

Pollen antigens. Pollen samples were defatted with diethyl ether and vacuum dried. Antigen was extracted in phosphate buffer saline (10% w/v) by continuous stirring at 4ºC for 20 hours. The extracts were centrifuged at 10,000 rpm for 30 min, and the supernatant was dialyzed against distilled water and lyophilized in small aliquots. The lyophilized samples were stored at -70ºC and reconstituted when and as required. The same set of antigens were used for all the experiments during the investigations.

Estimation of protein. Total soluble Protein was estimated using Lowry's method [12] with slight modification as protein was precipitated with Phosphotungestic acid and then dissolved in 2% sodium hydroxide solution. Micro-Kjeldhal technique was employed for estimation of protein nitrogen units (PNU) [10]. The mean protein/PNU concentration and co-efficient of variation (CV) were also calculated to see the extent of protein variability among five samples studied.

Iso Electric Focussing (IEF). IEF was performed on Ampholine PAGE plates (Pharmacia LKB Biotechnology, Sweden pH 3.5–9.5) as per manufacturer's manual. Antigen containing 100 microgram of protein was loaded with the help of small rectangular filter strips along with pI markers. The gel was stained with Comassie brilliant blue R 250 (Sigma USA). The method described by Garfin [7] was used for protein separation by IEF. IEF was carried out on Ampholine PAG plates (Pharmacia, Sweden) with pH gradient 3.5–9.5 (broad range) according to the manufacturer's manual. Extracts with varying protein concentrations (20–200 µg) were electro-focussed to determine the optimum concentration of protein required for optimum separation. The extracts were electro - focussed at constant voltage of 100 V for 15 min, followed by another 15 min at 200 V, and 1.5 hrs at 450 V. The optimum protein to be loaded was standardized to 100 µg for better separation. Proteins with known isoelectric points between 3.5 to 9.3 (pI markers) were electro-focussed along with the samples. After electro-focussing, the gel was fixed in a fixative solution (11.4% trichloroacetic acid and 3.4% sulphosalicylic acid) for 1 hr minimum and then washed with destaining solution (25% ethanol in 80% glacial acetic acid) for 30 min. The gel was then stained with Coomassie brilliant blue R 250 (0.13% in destaining solution) for 3 hrs. It was then destained till clear bands were obtained. The gels were photographed and then preserved.

Sodium dodecyl sulphate polyacrylamide gel eletrophoresis (SDS-PAGE). The procedure outlined by Laemmli [11] was followed. SDS-PAGE was carried out using polyacrylamide gel (12%) containing 0.1% SDS in conjunction with tris-glycine buffer (0.05 M tris, 0.384 M glycine, 0.1% SDS) using Mini Electrophoretic Apparatus (Bio Rad). Extracts with varying protein concentrations

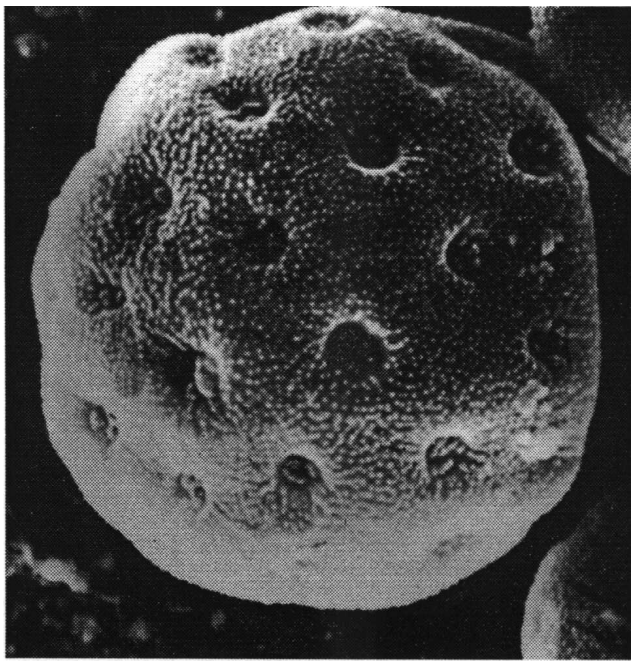

Figure 2. Pollen grain of *Amaranthus spinosus* showing pantoporate, spheroidal and scabrate surface under SEM (× 2500).

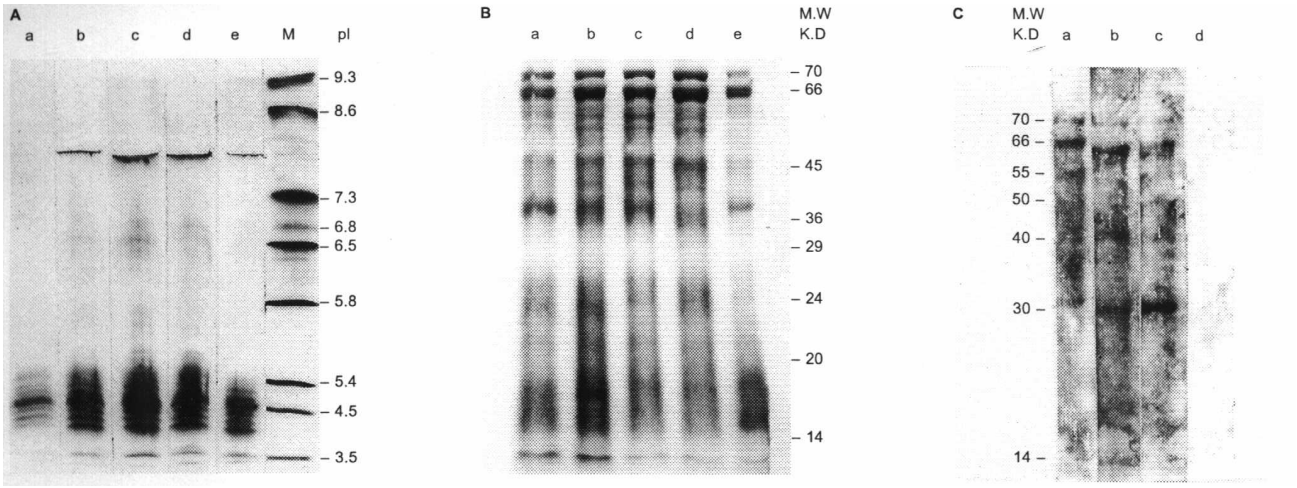

Figure 3. A. Isoelectric Focussing pattern (IEF) of antigenic extracts of five *Amaranthus spinosus* pollen samples (a, b, c, d, e) collected at fortnightly intervals during the same flowering season. **B.** SDS-PAGE protein profile of five *Amaranthus spinosus* pollen samples (a, b, c, d, e) collected at fortnightly intervals during the same pollination season from Delhi. **C.** Immunoblot showing *Amaranthus spinosus* specific IgE binding fractions of antigenic extracts (a, b, c) when probed with the sera of skin and RAST positive patients of respiratory allergy and control sera (d).

(20–200 µg) were used for standardization of the amount to be loaded for achieving separation of optimum number of bands. 20 µl of the sample containing 100 µg of protein in an equal amount of sample buffer (2% SDS; 10% glycerol; 5% β-mercaptoethanol; 0.01 M tris, pH 8.0; 0.001 M EDTA and 0.1 M bromophenol blue) was heated at 100ºC for 2 min to denature the proteins. After cooling, the samples were loaded on to the wells. The gels were calibrated with marker proteins with molecular weights of 14.2, 20.1, 24, 29, 45 and 66 kD (Sigma, USA).

Electrophoresis was carried out at a constant current of 4 mA / well. When the tracking dye in the gel reached 1 cm above the lower end of the gel, electrophoresis was terminated. The gel was stained overnight by keeping in the staining solution (10% glacial acetic acid and 0.25% Coomassie Brilliant blue in 95% ethanol). The gel were then destained in 5% glacial acetic acid and 95% ethanol for varying periods till protein bands appeared clear. After destaining, the gels were photographed and preserved.

Skin test. Intradermal skin tests were performed on 92 patients suffering from the naso-bronchial allergy, attending OPD of the V. P. Chest Institute. Approximately 0.01–0.02 ml of antigen was intradermally injected on the pre-cleaned volar surface of the forearm and anterio - lateral part of the upper arm to make a 2–3 mm wheal. Phosphate buffer saline and histamine hydrochloride (100 µg/ml) were also injected as negative and positive control respectively. In addition to atopic patients, skin test was also performed on 20 healthy non-atopic volunteers, to act as additional control. The skin response was observed after 15–20 min of the test and graded as per the criteria laid by Shivpuri [19].

Serum samples. Venous blood was drawn from skin test positive patients and sera were separated by centrifugation and stored at -70ºC in small aliquots for further use. Blood samples from 15 healthy volunteers were also collected to act as control.

RAST. Radioallergosorbent Test (RAST) was used to estimate *Amaranthus spinosus* specific IgE antibodies. Pollen allergen discs were prepared by the method of Gleich and Yunginger [8]. First, the discs (Whatman filter paper No. 50) were activated by cyanogen bromide and then coupled to these activated discs by continuos shaking at 4ºC for 72 hrs. The assay for estimation of specific IgE was carried out as per manufacturers manual (Pharmacia, Sweden). RAST positive sera from 12 patients were pooled to study the allergenic fractions of pollen antigens of *A. spinosus* extracts.

IMMUNOBLOT

Eletrophoretic transfer of proteins to nitrocellulose (NC) membranes. Protein separated by SDS - PAGE were electrophoretically transferred to 0.45µm NC for 5 hours, using Tris glycine buffer at 30 mA at 4ºC as per the method of Towbin *et al.* [21]. The unreacted sites after transfer were blocked with 3% BSA in TPBS at room temperature. Immunoblotting was carried out to determine the IgE binding fractions in pollen extracts of Himalayan trees, and also to study the variation in the allergen binding profile of RC pollen before and after exposure with gaseous pollutants. Highly positive sera (showing high binding with specific antigens detected by ELISA) from hypersensitive patients were used. Protein bands, separated onto SDS-PAGE were electrophoretically transferred to the NC membranes following the method of Towbin *et al*. [21]. NC membranes (0.45 µm) of the size of the gel were cut and soaked in transfer buffer (25 mM tris, 192 mM glycine, 20% methanol, pH 8.3) an hour before the transfer of proteins. Proteins were then blotted to NC membranes by electro transfer, using Tris glycine buffer (pH 8.3). After blotting for 5 hrs, unreacted sites on the NC membranes were blocked by incubating them with 3% BSA in 0.5% TPBS for 3 hrs at room temperature (22–24ºC). Membranes were then washed 4 times with

Table 1. Protein content of *Amaranthus spinosus* antigens from pollen collected of frequent intervals from Delhi during the same flowering season (July-Sept).

Samples	Protein mg/ml	PNU/ml
a	0.76	12,600
b	0.91	14,200
c	0.78	14,000
d	0.60	12,900
e	0.40	10,500
CV	25%	10%

Table 2. Skin test result conducted with *Amaranthus spinosus* antigens from pollen collected at different intervals on 92 patients.

Sample No	1+ to 4+		2+ to 4+		3+ & above	
	No	%	No	%	No	%
a	28	30.4	21	22.8	2	2.2
b	35	38.0	8	8.7	7	7.6
c	30	32.6	14	15.2	8	8.7
d	35	38.0	10	10.8	8	8.7
e	33	35.8	10	10.8	9	9.7

washing buffer of 0.05% TPBS. The NC membrane strips for each sample were cut and incubated with pooled sera of ELISA positive cases. Pooled sera from healthy volunteers showing negative skin reactivity and ELISA (pooled from individuals) were used as control.

To see the heterogeneity in specific IgE binding, protein fractions as recognized by sera from individual patients of some Himalayan pollen extracts, membranes were incubated with positive individual patient's sera. In all the incubations, the serum was diluted in the ratio of 1 : 5 using PBS containing 0.05% Tween 20. The strips were washed thoroughly, as stated earlier. After washing, the strips were incubated with antihuman IgE peroxidase conjugate (Sigma, USA) in the ratio of 1 : 1000 in 0.03% TBPS for 3 hrs. These strips were again washed thoroughly and placed in DAB (10 mg of 3–3 Diaminobenzidine in 20 ml of PBS containing 30 µl of hydrogen peroxide) solution in dark for colour development for 5 min. After treatment with 0.2 N hydrochloric acid, the strips were washed with distilled water and stored in the dark.

Probing of NC membranes. The NC strips were washed thoroughly with 0.5% TPBS and incubated (26 hrs) with RAST positive sera (1 : 10) diluted with PBS containing 0.1% sodium azide. After washing, the strips were incubated with antihuman IgE peroxidase conjugate diluted to 1 : 500 with 0.3% TPBS for 3 hours. The strips were washed again and incubated with substrate solution in the dark (10 mg of 3–3 diaminobezidine in 20 ml of PBS with 30 µl of hydrogen peroxide) for color development. Developed strips were treated with distilled water containing 0.2N HCl for 2–3 minutes.

RESULTS

Protein content. The protein concentration and the protein nitrogen units of extracts prepared from pollen obtained at different intervals are given in Table 1. Their concentration varied from 0.40 mg/ml to 0.91 mg/ml (mean 0.69 ± 25% (U) for soluble protein and 10500 to 14200 (mean 12840 ± 10% (U)) for PNU, respectively. The CV for soluble protein was 25% while that of PNU was 10%.

Iso Electric Focusing (IEF). IEF patterns of the extracts collected at different times during the same pollination season did not exhibit marked variation, as can be seen from Figure 3A. The majority of the 24 protein bands are in the acidic range (pH 4.5–5.6) and only a few in the basic range. However, the fresh pollen samples collected at the start of the flowering season in July had lower number of bands, while pollen samples collected at the peak of the flowering seasons showed a larger number of bands. A few protein bands were not clearly visible in the 5th sample (e) collected in September.

Sodium dodecyl sulphate polyacrylamide gel eletrophoresis (SDS - PAGE). The protein profile of the different samples of *A. spinosus* seems almost identical with 22 fractions (Fig. 3B). 22 bands in the mw wt range of 14–70 KD were detected. The most prominent bands were 70, 66, 60, 50, 45, 36, 16 and 14 KD, detected in the extracts of the pollen collected during the peak flowering season. As in the case of IEF, the number of bands were also fewer in the sample collected in the beginning of the main flowering season and at the end, respectively.

Skin test. The results of the interadermal skin test conducted with the antigens of 5 pollen samples of *A. spinosus* on all the 92 hypersensitive naso-bronchial allergy patients are shown in Table 2. Marked positive skin reactions (2+ and above) with pollen extracts of different samples varied from 8.7% to 22.8% with samples b and a, respectively. However, total positivity (1+ to 4+) varied from 30.4–38.0% in the patients tested. None of the normal volunteers tested showed any positive response. However, 3+ and 4+ skin reactivity was higher in cases with pollen samples (c & d) collected during peak pollination season.

Immunoblot. Specific IgE binding fractions probed against the pooled sera from 12 atopic patients are shown in Figure 3C. The electrophoretic mobility of allergenic bands as seen on NC membrane was almost the same in all samples. Seven IgE binding protein fractions of 70, 66, 55, 50, 40, 30 and 14 KD were detected from the blot. However, when the pooled sera of normal volunteers were used no IgE binding fractions was observed.

DISCUSSION

A. spinosus grows as a weed along road sides and waste places in different parts of the country and is also widely distributed in Europe and the USA . High concentrations of its pollen in the atmosphere, along with high skin reactivity in the atopic patients, suggest that it is an important aeroallergen. Five pollen samples of *A. spinosus* pollen collected at different time intervals did not show appreciable variation in the protein and PNU content (CV ± 25%) and (CV ± 10%). This indicates that protein content in the samples from bloomed inflorescence did not vary much. Similar was the observation when the extracts were subjected to iso electric focusing and sodium dodecyl polyacrylamide gel electrophoresis. All the samples exhibited similar protein profile, identifying 24 bands in the pI range of 3.5–9.3. An almost identical pattern with 22 bands in the mw range of 14–70 KD was recorded in SDS-PAGE in the pollen collected during the same season. However, fewer bands were observed in the sample collected at the start of the main season and towards the end. This indicates that with the development of the anther, protein content also increases slightly. This is not strictly in accordance with our earlier observation with respect to *Ricinus communis* pollen where increased protein concentration was observed [20]. The allergenic reactivity of the extract as determined by the interadermal skin test also did not exhibit appreciable variation (CV ± 8.8%), which again showed similarity in the allergenicity of different extracts of *Amaranthus* pollen collected from bloomed inflorescence during the same season. The IgE binding pattern of 3 samples was also identical. Seven allergenic protein of 70, 66, 60, 50, 40, 30 and 14 KD were observed on the NC membrane when probed with sera of the RAST positive cases with pollen samples studied.

Scientists from other parts of the world have shown both batch to batch and seasonal differences and similarities for *Cynodon dactylon*, *Dactylis glomerata*, *Lolium perenne* and *Phleum pratense* [4, 5, 6]. Our results are conform with those of the Russian thistle pollen extract where similarities among different batches have been reported [15]. It is therefore concluded that the different pollen samples of *A. spinosus* collected from similar stage of flowering during the same season did not show any appreciable variation in their antigenic and allergenic properties. We recommend that the pollen should be collected from the flowers of the same stage from the same locality for antigen preparation, for diagnosis and immunotherapy.

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