Research Article

ALLERGENICITY OF DOMINANT AEROPOLLEN IN NIGERIA: PART I

Tiwalade Adeyemi Adeniyi
Peter Adegbenga Adeonipekun
James Dele Olowokudejo

Department of Botany, University of Lagos, Nigeria

Email | dessytwa@yahoo.com

ABSTRACT

Several pollen grains have been studied in detail for purification and characterisation of allergenic components in the advanced countries; however, many are yet to be studied in the tropics, including Nigeria. To close this gap, four pollen grains (Poaceae – *Cynodon dactylon, Panicum maximum;* Cyperaceae – *Cyperus rotundus* and *Mariscus alternifolius*) found dominant in the air from previous aeropalynology studies in Nigeria were selected for allergenicity studies. The pollen grains were harvested from fresh anthers and their proteins were extracted, quantified, separated, subjected to Western-blot analysis, and allergenic proteins were identified. *C dactylon* had the highest protein content (17.09 mg/mL), whereas *M alternifolius* had the lowest (11.19 mg/mL). Western-blot analysis showed that individuals were most susceptible to the 35 kDa protein of *C dactylon* (76%). Furthermore, only *C dactylon* proteins of 14.5 kDa and 35 kDa were identified with their exact matches in the ProFound database (Cyn d 12 and Cyn d 1 respectively), whereas the peptide sequences of eight protein bands were newly added to the database. Of these, the Profilin protein group (14 kDa) is common to all studied pollen grains – an indication of veritable immunotherapeutic potential. This study is the first in Nigeria to record allergenic proteins in these pollen grains and to create a foundation for the development of immunotherapy drugs for allergy treatment in the country.

Keywords: allergenic proteins; immunotherapy; Nigeria; pollen grains; Western blot

INTRODUCTION

It is often difficult to believe that the seasonal catarrh or wheezing cough or 'apollo' (conjunctivitis) is merely an abnormal condition of our immune system. Allergic individuals will produce immunoglobulin E (IgE) upon first contact with an antigen, termed an 'allergen'. Upon re-exposure, IgE binds to the IgE-specific receptors (Fc epsilon receptors) of mucosal and cutaneous mast cells and circulating basophils.[1] This reaction occurs within minutes, and upon re-exposure to the allergen, there is a rapid uptake of calcium ions into the mast cells; this results in degranulation and the release of pro-inflammatory mediators such as histamine, leukotrienes, prostaglandins and tryptase.[1] These, in turn, result in the symptoms of immediate allergic reactions. Allergenic conditions are characterised by intense sneezing (rhinitis), watery eyes, nasal obstruction, itchy eyes and nose, wheezing, coughing and asthma.[1] Other organs reacting to allergenic attacks – according to Kay[1] – are skin (urticaria), gastro-intestinal tract, central or peripheral nervous system and cardio-vascular system. Allergens are antigens that stimulate allergic reaction.[2] Mites, fungi and endothelial tissues/dander from pets are common indoor allergens, whereas for outdoor allergens, pollen and fungal spores dominate.[3] According to Ziello et al,[4] more than 250 000 well-described pollen-producing plants exist but fewer than 100 represent potent sources of allergens. Pollen allergens belong to various protein families that have been identified in diverse plant species mainly in the temperate regions.[5,6,7,8,9]

Pollens causing allergy are quite variable in different ecozones, which makes it very important to identify allergy-causing species from every region and to prepare extracts from them for diagnosis and immunotherapy for the benefit of allergy sufferers. Although allergenic protein characterisation and identification is rare in Nigeria, aerobiological studies have revealed some dominant pollen grains in the air which could be allergenic due to their abundance and the plant families they belong to. Examples of these dominant pollen grains are grasses and sedges.[10,11,12,13] This dominance is due to the cosmopolitan

nature of this group of plants in Nigeria: they are found in gardens, fields, drainages, river banks, cleared lands, etc.

Common grass species in Nigeria include: *Cynodon* spp, *Panicum* spp, *Axonopus compressus, Pennisetum* spp, *Paspalum* spp, *Andropogon* spp, *Sorghum* spp, *Triticum* spp, *Zea mays, Oryza* spp and *Stylosanthes* spp.[14]

Common sedges in Nigeria are: *Cyperus* spp, *Carex* spp, *Kyllinga* spp, *Marisucs* spp and *Pycreus* spp.[14]

Furthermore, the pollen grains of grasses are all similar – spherical and monoporate.[15] It is often difficult for palynologists to differentiate between the pollen of grass species. Similarly, the pollen grains of sedges which are often in tetrads or monads split from tetrads are very alike.[15] Palynologists therefore group these identical pollen grains under their respective families – Poaceae for grasses and Cyperaceae for sedges. However, in allergenicity studies, a vegetation reconnaissance of the sampled area is conducted to determine the dominant species within the region. The pollen of such dominant species can then be collected for analysis. Since the majority of the aerobiology research in Nigeria has been conducted in the southern part of the country,[10,11,12,13] this research selected grasses (*Cynodon dactylon* (L) Pers and *Panicum maximum* Jacq) and sedges (*Cyperus rotundus* (L), *Mariscus alternifolius* Vahl) common to southern Nigeria.[14]

As explained above, several pollen grains have been studied in detail for purification and characterisation of allergenic components in the advanced countries; none are yet to be studied in Nigeria. This has led to misdiagnosis and self-medication of many allergy sufferers in the country. In view of this problem, the characterisation and identification of the allergenic proteins in dominant pollen grains in Nigeria are necessary for diagnosing and treating allergies.

MATERIALS AND METHODS

POLLEN HARVESTING

Anthers of *C dactylon, P maximum, C rotundus* and *M alternfolius* were collected and dried at room temperature and then converted to powder form by being crushed with a glass mortar and pestle. The powder was filtered through 200, 300 and 400 µm mesh sizes to collect fresh pollen and sieve out anther and other floral components.

PROTEIN EXTRACTION

Pollen grains were incubated and rotated overnight in phosphate buffer saline (PBS) to extract water-soluble proteins and in detergents (SDS 4%) to extract non-water-soluble proteins. After a centrifugation at 20 000 g at 4°C for 15 minutes, supernatants were collected and stored at −20°C until used. The protein concentration was measured using the classical Bradford method.[16] Here, protein standard, bovine serum albumin (BSA) was diluted with 0.15 M NaCl to final concentrations of 0 (blank = NaCl only), 250, 500, 750 and 1 500 µg/mL. Serial dilutions of each pollen sample were also prepared. One hundred microlitres of each sample were added to a spectrophotometer tube and 5.0 mL of Coomassie Blue was added to each tube; the mixture was then vortexed. Spectrophotometric readings were taken at a wavelength of 595 nm and blanked using the tube which contained no protein. The absorbance of the standards versus their concentration was plotted and the protein content of the pollen samples was computed from the extinction coefficient.

PATIENTS' SERA

Blood samples were collected from 50 allergy patients with a history of allergies and asthma from Gbagada General Hospital, Gbagada, Lagos State after an approval from the Ethics Committee of the hospital. The patient's symptoms and biological data were documented (gender, age group and allergic conditions). The criteria for including allergy patients comprised:

- a history of allergies;
- the timing and seasonality of allergies;
- age between 18 and 65;
- the allergic condition is not life-threatening;
- the patient is Nigerian by nationality and resides in Nigeria.

Patients were excluded if they exhibited one or more of the following exclusion criteria:

- no records of allergy history;
- allergies' seasonality or timing unknown;
- do not fall between ages of 18 and 65;
- the allergic condition is life-threatening;
- the patient is not Nigerian or is Nigerian but not resident in Nigeria.

For each analysis, the sera from five healthy individuals were used as controls. The healthy patients were selected from volunteers who donated blood to the hospital. Blood collection was done by a medical expert using syringes and anticoagulant tubes. The tubes were inverted eight times immediately after collection to distribute the additive evenly. Centrifugation was done immediately at 1 300–1 800 g for 10–15 minutes. The cell-free supernatant plasma (serum) was removed carefully using a syringe. The extracted serum was placed in a properly labelled polypropylene vial and stored at 4°C in sodium azide (0.02%) (Ramavovololona et al 2013)[23] until used.

GEL ELECTROPHORESIS

Proteins from pollen extracts were separated according to their relative mass (Mr) in SDS-PAGE. SDS-Polyacrylamide gel electrophoresis was performed in 12% gel according to the method of Laemmli[17] with modifications from Mondal et al.[18] Ten microlitres of the protein sample was heated with an equal amount of sample buffer (0.06 M is HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% β-mercaptoethanol,

0.01% Bromophenol Blue) at 100 °C for three minutes and loaded into the well of the gel; electrophoresis was then performed using a Laemmli buffer system (0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4) at room temperature and 70 V for two-and-a-half hours. The gel was calibrated with a marker mixture consisting of myosin from rabbit muscle (MW 205 kDa), β-galactosidase from *Escherichia coli* (MW 116 kDa), phosphorylase from rabbit muscle (MW 97.4 kDa), albumin from bovine (MW 66 kDa), albumin from egg (MW 45 kDa) and carbonic anhydrase from bovine rrythrocytes (MW 29 kDa) obtained from the Sigma Company, United States. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R 250 mixture to take photographs before being blotted onto a cyanogen-bromide-activated nitrocellulose (NCa) membrane for further Western-blot.

WESTERN-BLOT

Protein bands obtained by SDS-PAGE were transferred by semi-dry blotting (45 minutes, 0.8 mA/cm^2) onto nitrocellulose membranes. The membranes were cut into strips, washed with 0.05% Tris-buffered saline-Tween buffer (TBST: 50 mM Tris, 150 mM NaCl pH 7.4, 0.05% Tween20) for five minutes at room temperature (25 ºC), blocked twice with 0.3% TBST for 30 minutes, and washed again. They were then incubated overnight in a 1:10 dilution of sera from patients (n = 50) and controls (n = 5). The strips were subsequently washed four times with 0.05% TBST buffer and incubated with a 1:750 dilution of goat antihuman IgE-alkaline phosphatase (AP) for three hours. Antibody binding was detected by staining with the chromogenic substrate nitro-blue tetrazolium, 5-bromo-4-chloro-3'-indolyphosphate.[19]

IDENTIFICATION OF ALLERGENS

The spots of interest were analysed using the peptide-mass fingerprinting method.[20] Protein spots were excised from a Coomassie blue-stained gel. Proteins were then reduced with di-thio-threitol, alkylated with iodoacetamide and in gel-digested with porcin trypsin. The resulting peptides, in solution, were added to the matrix a-cyanohydroxy-cinamic acid and submitted to laser shot in a mass spectrometer (Applied Biosystem Voyager DE-STR instrument, Texas, United States). The instrument was calibrated using two trypsin autodigestion peaks (m/z 842.5099 and m/z 2211.1045) to draw a calibration curve. The tolerance was adjusted from 4 to 500 ppm. Peptide listings were then submitted to databases using a ProFound proteomic tool (ExPaSy Proteomics Server).

RESULTS

PROTEIN CONTENT

Cynodon dactylon had the highest protein content (17.09 mg/mL), whereas *M alternifolius* had the least (11.19 mg/mL) (see Table I).

TABLE I: PROTEIN CONTENTS OF POLLEN

POLLEN	PLANT FAMILY	COMMON NAME	PROTEIN CONTENT (mg/mL)
C dactylon	Poaceae	Bermuda grass	17.09
C rotundus	Cyperaceae	Tiger nut sedge	14.25
M alternifolius	Cyperaceae	Umbrella sedge	11.19
P maximum	Poaceae	Guinea grass	12.47

GEL ELECTROPHORESIS

The protein profile of *C dactylon* pollen showed five major bands in the molecular weight range of 14.5–55 kDa. *M alternifolius* pollen showed two protein bands at 14 kDa and 40 kDa. The protein profile of *C rotundus* pollen showed five major bands with a molecular weight range of 14–58 kDa. *Panicum maximum* pollen showed five protein bands with a molecular weight range of 14–58 kDa (see Figure 1).

WESTERN-BLOT

Three dominant protein fractions of 55 kDa, 35 kDa and 14.5 kDa in *C dactylon* pollen extract were observed to indicate a 50% (25 individuals), 76% (38 individuals) and 22% (11 individuals) IgE-binding capability. In *M alternifolius,* 14 kDa protein indicated a 32% (16 individuals) while 40 kDa indicated a 1% (two individuals) IgE-binding capability. Three dominant protein fractions of 55 kDa, 37 kDa and 14 kDa in *C rotundus* pollen extract were observed to indicate 26% (13 individuals), 10% (five individuals) and 12% (six individuals) IgE-binding capabilities respectively. Three dominant protein fractions of 55 kDa, 35 kDa and 14 kDa in *P maximum* pollen extract

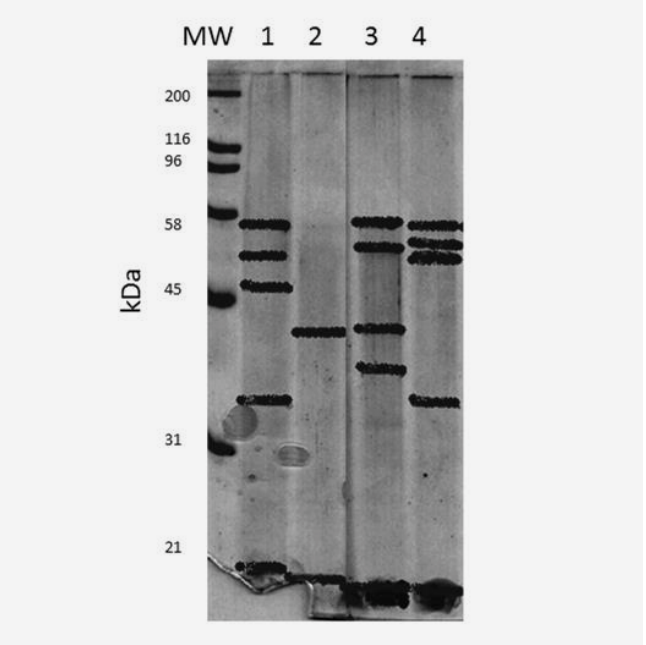

Figure 1: Protein profiling of different pollen types in 12% denaturing gel following SDS PAGE; 1 – C dactylon, 2 – M alternifolius, 3 – C rotundus, 4 – P maximum

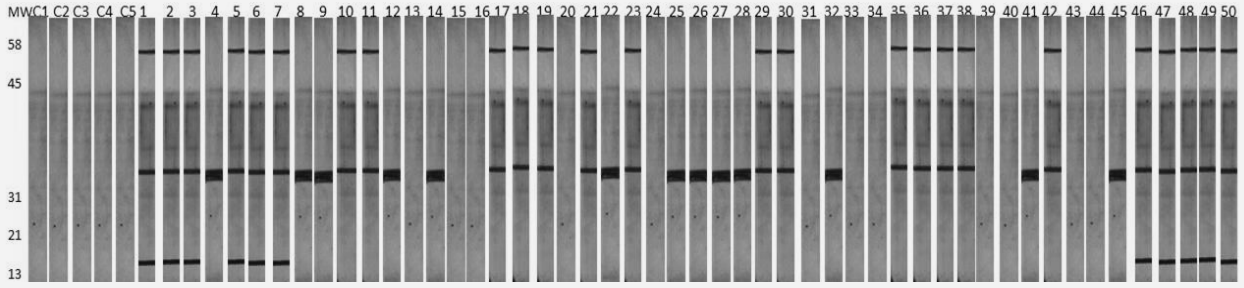

Figure 2a: Immunoblotting of C dactylon pollen extracts. IgE-reactive protein bands are darker. MW = Molecular weight. C1–5 = Controls, non-hypersensitive individuals. Lanes 1–50 = Sera of hypersensitive individuals

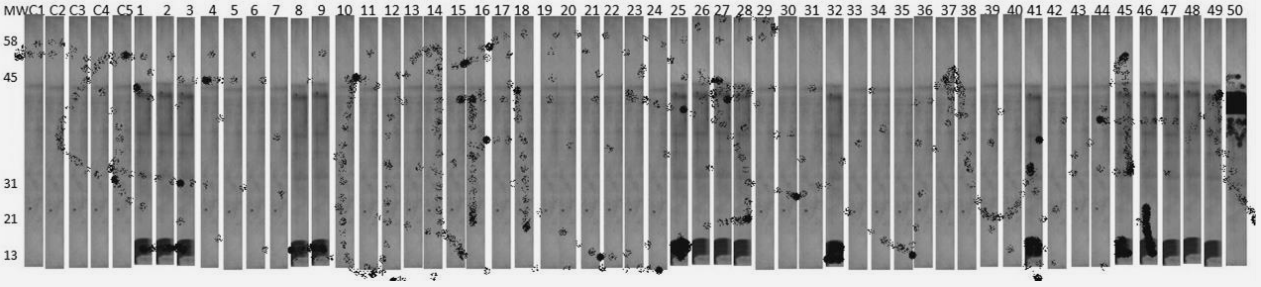

Figure 2b: Immunoblotting of M alternifolius pollen extracts. IgE-reactive protein bands are darker. MW = Molecular weight. C1–5 = Controls, non-hypersensitive individuals. Lanes 1–50 = Sera of hypersensitive individuals

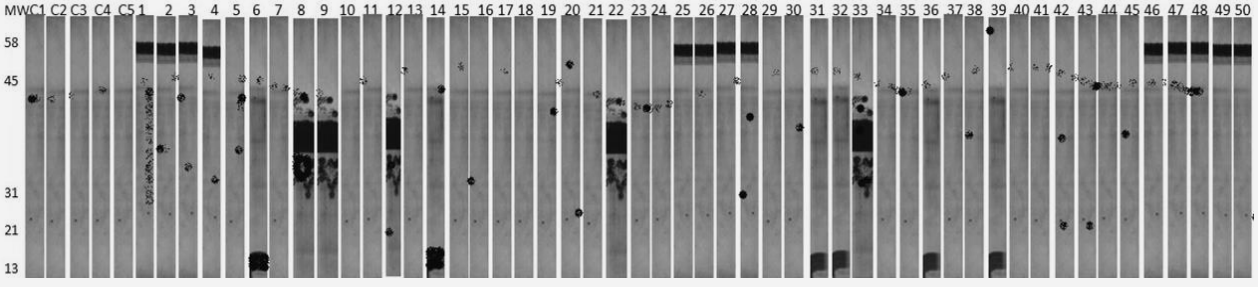

Figure 2c: Immunoblotting of C rotundus pollen extracts. IgE-reactive protein bands are darker. MW = Molecular weight. C1–5 = Controls, non-hypersensitive individuals. Lanes 1–50 = Sera of hypersensitive individuals

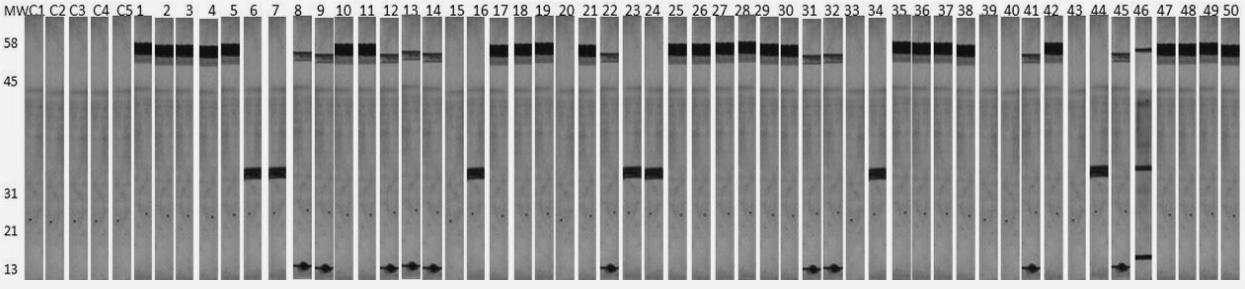

Figure 2d: Immunoblotting of P maximum pollen extracts. IgE-reactive protein bands are darker. MW = Molecular weight. C1–5 = Controls, non-hypersensitive individuals. Lanes 1–50 = Sera of hypersensitive individuals

TABLE II: ALLERGEN IDENTIFICATION

POLLEN	PROTEINS			
	PROTEIN (DA)	NAME	HOMOLOGUE ALLERGEN	PLANT ORIGIN
C dactylon	55 000	Polygalacturonase	Phl p 13	*P pratense*
	35 000	Beta-expansin	Cyn d 1	*C dactylon*
	14 500	Profilin	Cyn d 12	*C dactylon*
M alternifolius	14 000	Profilin	Phl p 12	*P pratense*
C rotundus	55 000	Polygalacturonase	Phl p 13	*P pratense*
	37 000	Pectin methylesterase	Ole e 11	*O europaea*
	14 000	Profilin	Phl p 12	*P pratense*
P maximum	55 000	Polygalacturonase	Phl p 13	*P pratense*
	35 000	Beta-expansin	Zea m 1	*Z mays*
	14 000	Profilin	Zea m 12	*Z mays*

were observed to indicate a 74% (37 individuals), 16% (eight individuals) and 22% (11 individuals) IgE-binding capability respectively (see Figure 2)

IDENTIFICATION OF ALLERGENS

All the protein bands, except the 40 kDa protein band of *M alternifolius*, were linked to homologues. Only *C dactylon* proteins of 14.5 kDa (profilin) and 35 kDa (beta expansin) were identified with their exact matches in the database (Cyn d 12 and Cyn d 1 respectively). Therefore, the newly sequenced eight allergenic proteins were deposited in the ProFound database with unique UniProt accession numbers. Profilin (14 kDa) was found in all the pollen sampled. However, the profilin of *C rotundus* and *M alternifolius* (both in the family Cyperaceae) were linked to allergen Phl p 12 of *Phleum pratense* while *P maximum* profilin was related to Zea m 12 of *Zea mays*, also a grass. The 55 kDa protein bands of *C dactylon, P maximum* and *C rotundus* were all polygalacturonases linked to allergen Phl p 13 of *Phleum pratense*. Also, *C dactylon* and *P maximum* had 35 kDa protein, which are beta-expansins and mainly related to Cyn d 1 and Zea m 1 respectively. The 37 kDa pectin methylesterase of *C rotundus* was related to Ole e 11 of the tree *Olea europaea* (see Table II).

DISCUSSION

Pollen grains contain proteins that can be allergenic. A particular pollen may contain several types of protein, all or some of which may be allergenic. Also, the susceptibility of allergy sufferers to each protein allergen is different. This supports the reason for diagnosis of each hypersensitive individual to ascertain the set of allergenic proteins they are susceptible to. Stanley and Linskens[21] reported that the protein level in pollen generally ranges between 5.9 and 28.3 mg/mL of pollen residue. Similarly, in this work, the protein levels of the pollen grains studied were within the range 11.19 mg/mL to 17.09 mg/mL.

This work is the first to document the protein profile of *C rotundus* and *M alternifolius.* Protein profiles of *C dactylon* and *P maximum* have been reported by other foreign authors. The protein profile of *C dactylon* pollen showed five major bands in the molecular weight range of 14.5–55 kDa, which was a similar range reported by Prescott and Potter[22] in South Africa and more recently by Ramavovololona et al[23] in Madagascar. *Panicum maximum* pollen also showed five protein bands with a molecular weight range of 14–58 kDa and this was a similar range reported by Ramavovololona et al[23] in Madagascar.

Qualitative observation of different immunoblots using a set of sera is very informative when no IgE tests for pollen taxa are commercially available, as is the case for three of the four plants in this study, the exception being *C dactylon*. In this report, 38 individuals of 50 (76%) were observed to show IgE-positivity with *C dactylon*. Ramavovololona et al[23] reported a similarly high percentage for *C dactylon*, with 27 of 39 (69%) individuals showing IgE-positivity. These results suggest that seven to eight out of ten hypersensitive individuals are susceptible to *C dactylon* pollen. Stemming from the fact that *C dactylon* is a grass and aa stubborn weed in the tropical and temperate regions, this high hypersensitivity warrants the control of the plant in gardens, parks, farms and drainages. In *P maximum*'s IgE results, 44 individuals of 50 (88%) were observed to show IgE-positivity to the pollen protein. However, Ramavovololona et al[23] reported a very low percentage for *P maximum*, with 13 of 64 (20%) individuals showing IgE-positivity. These results suggest that *P maximum* pollen hypersensitivity may be attributed to the season of pollen collection, the type of pollen protein and the individuals in the region. Nonetheless, the ubiquity of *P maximum* in Nigeria also warrants the plant being enclosed in gardens, parks, farms and drainages.

The work of Ramavovololona et al[23] failed to highlight the specific protein bands in *C dactylon* and *P maximum* that were responsible for the sensitivity. Here, 35 kDa protein of *C dactylon* and 55 kDa protein of *P maximum* were the most allergenic of the two grasses. This indicates the diverse sensitivity of the two grass taxa which originate from different sub-families.

The 35 kDa protein of *C dactylon* has been identified and registered in the database as a beta-expansin and named Cyn d 1. However, the 55 kDa of *P maximum* is a polygalacturonase still being linked to Phl p 13 of *P pratense.* This work is the first to map the highly allergenic protein in *P maximum* and register it in the database. Similarly to the Poaceae, the Cyperaceae plants *C rotundus* and *M alternifolius* are also stubborn weeds. Both pollen taxa exhibited low–mild allergenicity. However, the allergenic 14 kDa in both pollen taxa was similar to the Phl p 12 of *P pratense,* a Poaceae member. The similarity of a protein in the Poaceae to that of the Cyperaceae reaffirms several publications on the divergent evolution of both groups of plant from a common ancestral line.

The results of this investigation have identified allergenic proteins in the studied pollen which are often dominant in the air in Nigeria. The allergenic proteins can be used to develop immunotherapy drugs to aid allergy diagnosis and treatment in Nigeria. Of particular importance is the broad-spectrum protein (Profilin) present in all the studied pollen grains and, therefore, it has high immunotherapeutic potential.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

This article has been peer reviewed.

REFERENCES

1. Kay A. Overview of allergy and allergy diseases with a view to the future. British Medical Bulletin 2000;56(4):843–864.
2. Blumenthal M, Rosenberg A. Definition of an Allergen (Immunobiology). In: Lockey RF, Bukantz SC, editors. Allergens and Allergen Immunotherapy. New York: Marcel Dekker Inc., 1999;39–51.
3. Kerkhof M, Postma D, Schouten J, De Monchy J. Allergic sensitization to indoor and outdoor allergens and relevance to bronchial hyper-responsiveness in younger and older subjects. Allergy 2003;58:1261–1267.
4. Ziello C, Sparks T, Estrella N, Belmonte J, et al. Changes to airborne pollen counts across Europe. PLoS ONE 2012;7(4):e34076. doi:10.1371/journal.pone.0034076.
5. Di Felice G, Barletta B, Tinghino R, Pini C. Cupressaceae pollinosis: identification, purification and cloning of relevant allergens. Int Arch Allergy Immunol 2001;125:280–289.
6. Andersson K, Lidholm J. Characteristics and immunobiology of grass pollen allergens. Int Arch Allergy Immunol 2003;130:87–107.
7. Gadermaier G, Dedic A, Obermeyer G, Frank S, et al. Biology of weed pollen allergens. Curr Allergy Asthma Rep 2004;4:391–400.
8. Mothes N, Westritschnig K, Valenta R. Tree pollen allergens. Allergens and Allergen Immunotherapy. Curr Allergy Asthma Rep 2004;18:165–184.
9. Asam C, Hofer H, Wolf M, Aglas L, et al. Tree pollen allergens – an update from a molecular perspective. Allergy 2015;70:1201–1211.
10. Njokuocha R. Airborne pollen grains in Nsukka, Nigeria. Grana 2006;45(1):73–80.
11. Adeniyi TA, Adeonipekun PA, Olowokudejo JD, Akande IS. Airborne pollen records of Shomolu Local Government Area in Lagos State. Notulae Scientia Biologicae 2014;6(4):428–432.
12. Ezike DN, Nnamani CV, Ogundipe OT, Adekanmbi OH. Airborne pollen and fungal spores in Garki, Abuja (North-Central Nigeria). Aerobiologia (Bologna) 2016;32(4):697–707.
13. Adeonipekun PA, Agbalaya AE, Adeniyi TA. Aeropalynology of Ayetoro-Itele, Ota Southwest Nigeria: A preliminary study. In: Alabi R, Adeonipekun PA, editors. Human Palaeoecology in Africa: Essays in Honour of M. Adebisi Sowunmi. Ibadan, Nigeria: University of Ibadan Press, 2016;130–153.
14. Ayodele AE, Yang Y. Diversity and distribution of vascular plants in Nigeria. China: Qingdao Publishing House, 2012.
15. Erdtman G. Pollen Morphology and plant taxonomy, angiosperms. New York: The Ronald Press Company, 1952.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
17. Laemmli UK. Cleavage of structural protein during assembly of the head of the bacteriophage T4. Nature 1970;227:680–684.
18. Mondal S, Mondal AK, Mandal S. Evaluation of electroelution and immunodiffusion as methods for purification and identification of the allergenic proteins of Ailanthus excelsa Roxb. Pollen. Grana 2007;46(2):91–97.
19. Micheal S, Wangorsch A, Wolfheimer S, Foetisch K, et al. Immunoglobulin E Reactivity and Allergenic Potency of Morus papyrifera (Paper Mulberry) Pollen. J Investig Allergol Clin Immunol 2013;23(3):168–175.
20. Poncet P, Senechal H, Clement G, Purohit A, et al. Evaluation of ash pollen sensitization pattern using proteomic approach with individual sera from allergic patients. Allergy 2010;65:571–580.
21. Stanley R, Linskens H. Pollen biology, biochemistry and management. Berlin: Springer Verlag, 1974.
22. Prescott RA, Potter PC. Immunochemical characterisation of grass pollen allergens in South Africa. Curr Allergy Clin Immunol 2007;20(4):189–193.
23. Ramavovololona P, Senechal H, Ramamonjisoa ZR, Andrianarisoa ACF, et al. Allergy to pollen of six common grasses in the highlands of Madagascar. Curr Allergy Clin Immunol 2013;26(4):184–191.