Impacto do nível de urbanização no pólen de *Chenopodium album*: Morfologia e imunoquímica

Impact of urbanization level on Chenopodium album *pollen: Morphological and immunochemical data*

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Raquel Sousa[1], Ana Paula Cruz[2], Helena Ribeiro[1], Ilda Abreu[1,3*]

[1] Grupo Ambiente do Centro de Geologia da Universidade do Porto, Portugal
[2] Serviço de Patologia Clínica, Laboratório de Imunologia do Centro Hospitalar, Vila Nova de Gaia, Portugal
[3] Departamento de Biologia da Faculdade de Ciências da Universidade do Porto, Portugal

RESUMO

Introdução: O pólen de *Chenopodium album* é abundante na atmosfera no fim do Verão e Outono sendo, portanto uma fonte potencial de aeroalergénios. Há evidências de que a prevalência e a severidade de doenças atópicas têm vindo a aumentar nas áreas urbanas. **Objectivo**: Pretendeu-se estudar a estrutura, perfil proteico e potencial alergizante do pólen de *Chenopodium* recolhido em área urbana e rural, a fim de inferir o agravamento de sua alergenicidade devido ao grau de urbanização. **Métodos**: A morfologia polínica foi analisada ao microscópio electrónico de varrimento. As proteínas do pólen de *Chenopodium* foram separadas por SDS-PAGE, sendo o perfil analisado por *immunoblotting* usando soros de doentes polissensibilizados (wx1) e a ZmPRO3. **Resultados**: Foram observadas diferenças morfológicas entre o pólen colhido nos dois locais de estudo. No pólen recolhido em ambiente urbano os opérculos encontravam-se deformados e apresentavam partículas depositadas à sua superfície. Foram observadas diferenças no conteúdo em proteínas solúveis e no perfil de proteínas separadas por electroforese entre o pólen recolhido na cidade e em meio rural. Os ensaios de *immunoblot* com soros de doentes polissensibilizados (wx1) demonstraram marcação de vários componentes de ligação a IgE, alguns deles correspondendo a alergénios ainda não caracterizados, sendo a densidade óptica

das bandas reactivas superior para os extractos proteicos obtidos do pólen urbano em comparação com o rural. A utilização do anticorpo policlonal ZmPRO3 (profilina do milho) permitiu a identificação de uma proteína com peso molecular de 14 kDa. **Conclusões**: Este estudo indica que o ambiente urbano, com elevados níveis de poluição atmosférica, pode influenciar a alergenicidade do pólen.

Palavras-chave: Alergia, poluição atmosférica, quenopódio, *immunoblot.*

ABSTRACT

Introduction: Chenopodium album *pollen is one source of airborne allergens and is abundant in the atmosphere during late Summer and Autumn. There is evidence that the prevalence and severity of allergic diseases is increasing in urban areas.* ***Aim:*** *To study the structure, protein profile and allergenicity of chenopod pollen collected from urban and rural areas in order to analyse the increase in its allergenicity due to the urbanization level.* ***Methods:*** *Pollen morphology was analyzed by scanning electron microscopy. The pollen proteins extracted from the chenopod pollen collected from a urban and a rural area were separated by SDS-PAGE, and the allergenic profile was analysed by immunoblotting using sera from seven weed-polysensitized patients and maize profilin polyclonal antibody (ZmPRO3).* ***Results:*** *Morphological differences were observed between the urban and rural pollens, as the former showed deformed opercules and surface deposited particles. Urban chenopod pollen protein extract had a lower total soluble protein content and an SDS-PAGE pollen protein extract profile different from the rural one. Immunobloting tests showed labelling of various IgE binding components with different densitometric values, some of them corresponding to yet non-characterized allergens. All IgE binding proteins presented greater optical density values in the urban pollen protein extracts. The binding affinity of recombinant maize profilin 3 (ZmPRO3) polyclonal antibody to the* Chenopodium album *pollen protein extracts showed a band around 14 kDa.* ***Conclusions****: Our study indicates that urban environments with higher atmospheric pollution levels can influence airborne pollen allergenicity.*

Key-words: *Allergy, atmospheric pollution, chenopod, immunoblot.*

INTRODUCTION

Weeds represent a heterogeneous group of plants, usually without commercial or aesthetic value. Important allergenic weeds belong to plant families such as *Asteraceae, Chenopodiaceae-Amaranthaceae, Plantaginacea*e and *Urticacea*e. These weeds are usually wind-pollinated and shed large amounts of pollen locally.

Chenopod (*Chenopodium album,* commonly lamb's quarters) pollen is one source of airborne allergens in southern Europe, US and desert countries. In the Oporto region, its pollen is present in the atmosphere from late Summer until Autumn, and it is the most abundant pollen in the atmosphere during this period[1]. Frequently, patients allergic to chenopod pollen are polysensitized to other pollens[2-4]. Until now, *Chenopodium album* pollen allergens have been scarcely studied although three allergens have already been

characterized: Che a 1, Che a 2 and Che a 3[2, 5]. Che a 1 is a glycoprotein of 17 kDa with a sequence of 143 amino acid residues[2]. It is structurally related to Ole e 1-like protein family but with limited cross-reactivity and impaired antigenic activity, which could function as a primary allergy inductor instead[2]. Che a 2 and Che a 3 are relevant panallergens in chenopod pollen and may play a main role in its allergenicity. Che a 2 is a 14.4 kDa profilin with 131 amino acid chain and Che a 3 is a 9.5 kDa polcalcin with 86 amino acid residues[5]. Both display high IgE-binding affinity (prevalences) and cross-react with olive profilin (Ole e 2) and olive polcalcin (Ole e 3), respectively[3, 5, 6].

In Portugal, data regarding the prevalence of allergies specifically to *Chenopodium album* pollen is scant; however, in a previous study, based on 4915 positive skin prick tests from a specialized allergy clinic in Oporto, a 12% allergy prevalence to wx1 (weeds mixture which includes *Chenopodium album* – w10) was reported [7]. Also, in a profilin sensitization study in the Central region of Portugal, from 73 patients with positive skin prick tests to pollen around 39% were sensitized to *Chenopodium album* pollen[8].

There is evidence suggesting that respiratory allergic diseases related with atmospheric pollen content have been increasing in prevalence and severity in most industrialized and developing countries. To explain this increase, it has been proposed that air pollution can influence the way that pollen, once inhaled, is processed[9]. It has also been suggested that airway mucosal damage and impaired mucociliary clearance induced by air pollution may facilitate the access of inhaled allergens to cells of the immune system. In fact, when pollen is exposed to a variety of atmospheric substances, including air pollutants, it may show some modifications on its surface and biochemical properties that could lead to worsening of allergic symptoms in patients, or even to the development of symptoms in susceptible individuals.

The aim of the present work was to study the structure, protein profile and allergens of *Chenopodium album* pollen collected from urban and rural areas, in order to analyse the increase in its allergenicity due to urbanization level.

MATERIAL AND METHODS

Pollen samples

Pollen grains of *Chenopodium album* were collected during its flowering season from a rural area (coastal region of northern Portugal) and from an urban area with heavy traffic (city of Oporto).

After separation of extraneous materials, the anthers were dried at 27.°C, gently crushed and the pollen thus released was passed through different grades of sieves to obtain pure pollen. Pollen samples were then stored at –20 °C.

Pollen morphology analysis

For scanning electron microscopy (SEM) study, unacetolysed pollen grains were transferred to stubs, coated with gold film in the JEOL FC 1100 impregnator and examined with the FEI QUANTA 400 FEG ESEM/EDAX PEASUS X4M scanning electron microscope from CEMUP (Centro de Materiais da Universidade do Porto, Portugal).

Protein extraction and estimation

Dry pollen was suspended in 1:20 ratio in phosphate buffer saline at pH 7.4 (w/v) at 4 °C. Soluble proteins were extracted in the same buffer by continuous stirring for 4 h. The suspension was then centrifuged at 13 200 rpm for 30 min at 4 °C. The supernatant was then filtered through a 0.45 µm millipore filter and centrifuged once again. The soluble protein content of all the pollen extracts was quantified colourimetrically with the Coomassie Protein Assay Reagent (Pierce) by the Bradford method[10].

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli[11] in 12.5 % (w/v) polyacrylamide gels under reducing conditions and the proteins were visualized by Coomassie Brillant Blue R-250 staining. The molecular mass of protein bands was estimated by comparison with protein markers (Bio-Rad Laboratories). The antigenic profile was studied using a Molecular Imager GS800 calibrated densitometer

(Bio-Rad Laboratories) and Quantity One 1-D Analysis, v 4.6 (Bio-Rad Laboratories) software.

Immunoblots

Protein bands separated onto SDS-PAGE were electrophoretically transferred on a blot apparatus (TE22 Mighty small transphor unit – GE Healthcare) to a nitrocellulose membrane. Transfers were carried out in a solution of 192 mM glycine, 25 mM Tris and 20% methanol (v/v) (transfer buffer) during 2 h at 200 mA. The membranes were blocked during 45 min with 1% BSA (w/v), 0.1% goat serum (v/v), 5% non-fat dry milk (w/v) in TBS-T (20 mM Tris, 150 mM NaCl with 0.1% Tween 20) and incubated overnight at 4 °C on orbital shaking with rabbit polyclonal serum raised against recombinant maize profilin 3 (ZmPRO3) (Karakesisoglou *et al*. 1996) diluted 1:4000 and for the identification of allergens with atopic-patient sera diluted 1:8 in blocking buffer. After washing, membranes were probed with secondary antibody goat anti-rabbit IgG-HRP (Chemicon International) diluted 1:2000 in TBS-T and with mouse anti-human IgE-HRP (Southern Biotechnology Associates) diluted 1:2000 in TBS-T, during 1h at 4 °C on orbital shaking, respectively. The maize pollen profilin was expressed in *Escherichia coli*. Polyclonal antibody production was performed in a New Zealand White rabbit (Karakesisoglou *et al*. 1996). An ECL Western Blotting kit (GE Healthcare) was used as a detection system. The chemiluminescent signal was exposed to AGFA medical X-ray film and developed by Fugi medical film processor model FPM 100A, and the allergenic profile bands were quantified by a Molecular Imager GS800 calibrated densitometer with Quantity One 1-D.

Patient sera

Seven random patients previously selected as polysensitized to weed-mixture protein extracts (wx1 – *Ambrosia elatior, A. artemisilfolia, Artemisia vulgaris, Plantago lanceolata, Chenopodium album and Salsola kali*) assessed by skin-prick tests were chosen. Their sera had specific IgE values between low and very high (2.09 – 30.40 Ku/L). One negative control patient sensitized only to *Parietaria judaica* pollen – w21 (81.60 Ku/L) – was included in the study. Sera were separated from whole blood and allergen specific serum IgE were measured with a standard ImmunoCAP^{TM} test (ImmunoCAP^{TM} specific IgE, Phadia AB) and expressed as Kilo arbitrary unit/L (Ku/L), according to the manufacturer's instructions.

RESULTS

The scanning electron microscope study showed that *Chenopodium album* pollen grains are spheroidal, pantopolyporate with conical spinule opercules. Its exine is scabrate with

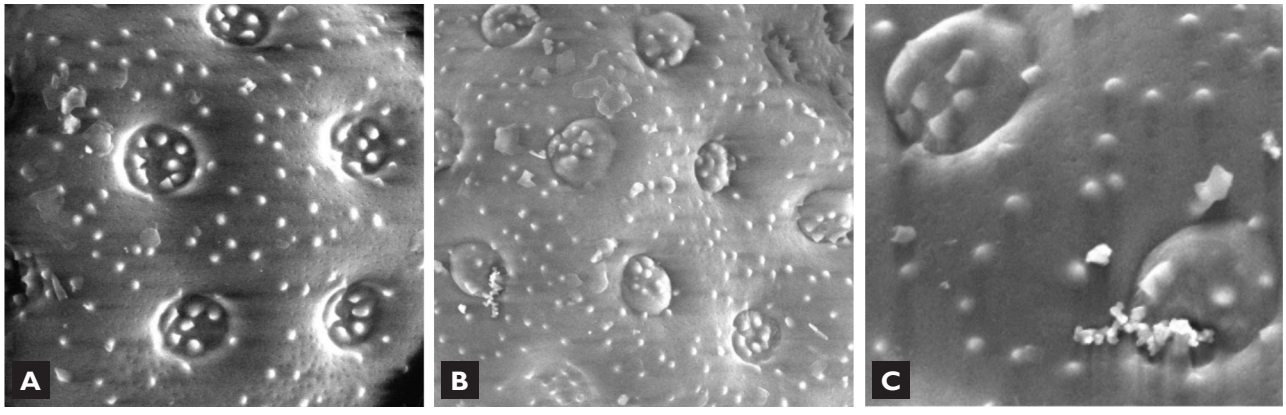

Figure 1. Scanning electron micrographs of *Chenopodium album* pollen grains. Pollen from the rural area (A) and from urban (B and C) area presenting deformed opercules (arrows) with particles deposition and a fine film covering the pollen wall when compared with the rural one. Bar 1 µm

some small conical spinules (Figure 1). Pollen opercules from the rural area are well defined (Figure 1A), unlike those from the urban area which are deformed with deposited particles and a fine film covering the pollen wall (Figures 1B and 1C).

When comparing pollens from urban and rural areas, some changes were found in protein content and their profile. The concentration of total soluble protein of urban pollen (4.60 mg mL^{-1}) was lower than the rural one (14.70 mg mL^{-1}).

The results of 12.5% SDS-PAGE of total soluble proteins from urban and rural *Chenopodium album* pollen extracts showed different protein profiles (as the urban protein bands revealed lower staining intensities of the bands and some were absent (Figure 2).

Chenopodium album pollen saline protein extracts probed against the sera of seven allergic patients, polysensitized to wx1, showed immunolabelling of various IgE binding bands with different densitometric values (Figure 3). All patients' sera analyzed presented reactivity to two bands with mo-

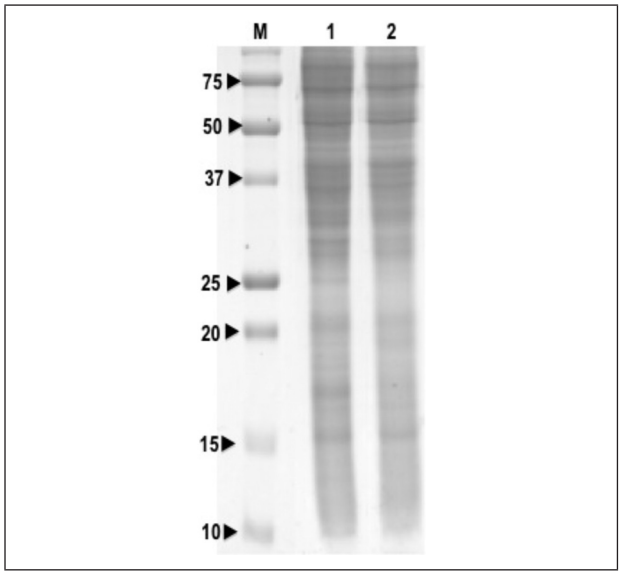

Figure 2. Coomassie Brilliant Blue-stained SDS-PAGE chenopod pollen protein extracts. Lane (1): rural pollen; lane (2): urban pollen and lane (M): molecular weight markers (kDa)

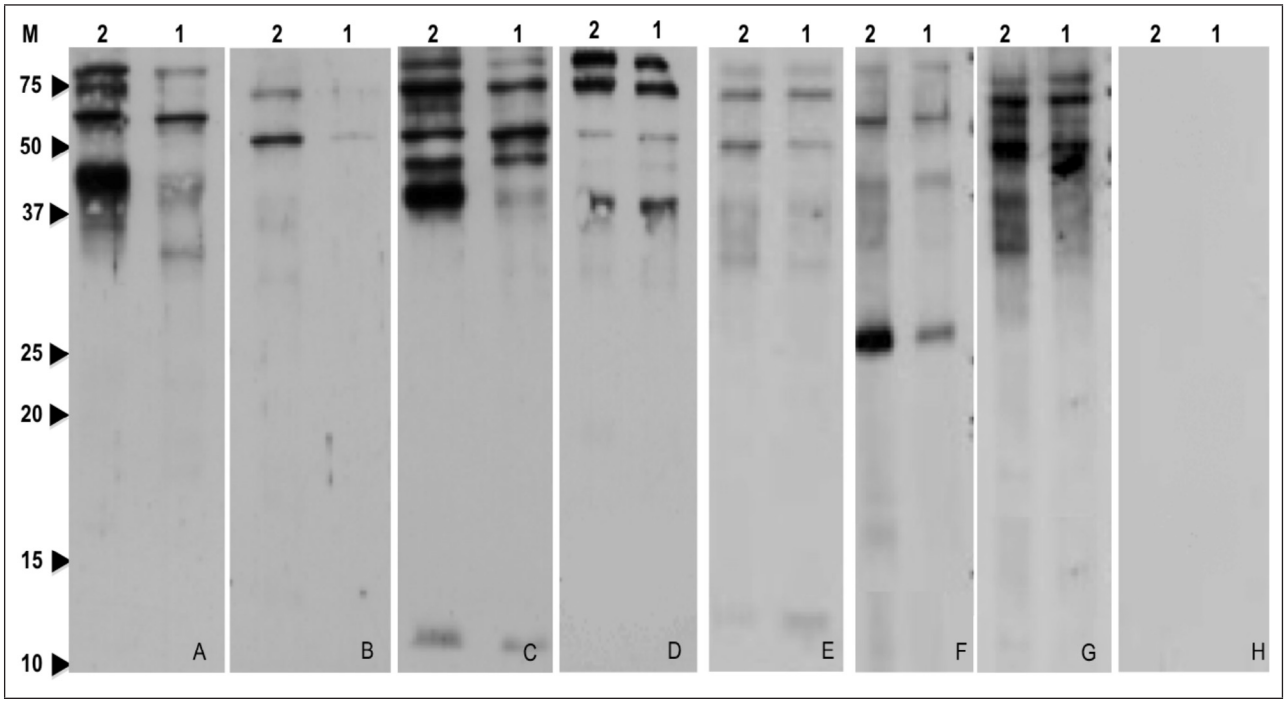

Figure 3. Immunoblots of chenopod pollen proteins incubated with polysensitized patient sera with specific IgE values (A: 5.87; B: 2.09; C: 30.40; D: 6.40; E: 2.13; F: 4.20; G: 12.60 kU/L) and control serum (H). Lane (1): rural pollen; lane (2): urban pollen and lane (M): molecular weight markers (kDa)

lecular weight around 70 and 53 kDa, in both urban and rural pollen extracts. Also, a band with molecular weight of about 40 kDa was present in five of the tested patient sera (A, C, D, F, and G). Below 15 kDa, we observed reactive bands in two patients' sera (C and E). Only one patient's serum presented reactivity to a protein band of molecular weight around 26 kDa (F). When the optical densities values of all these bands were compared, lower values in the rural pollen protein extracts were observed (Table 1). The negative control did not show significant labelling over the background (Figure 3).

All these assays were performed under identical conditions of signal development (time of exposure to X-ray film) in order to compare the relative intensities of the response in the IgE binding from different sera.

Results of binding affinity of recombinant maize profilin 3 (ZmPRO3) polyclonal antibody to the *Chenopodium album* pollen protein extracts showed the presence of one single band of about 14 kDa (Figure 4).

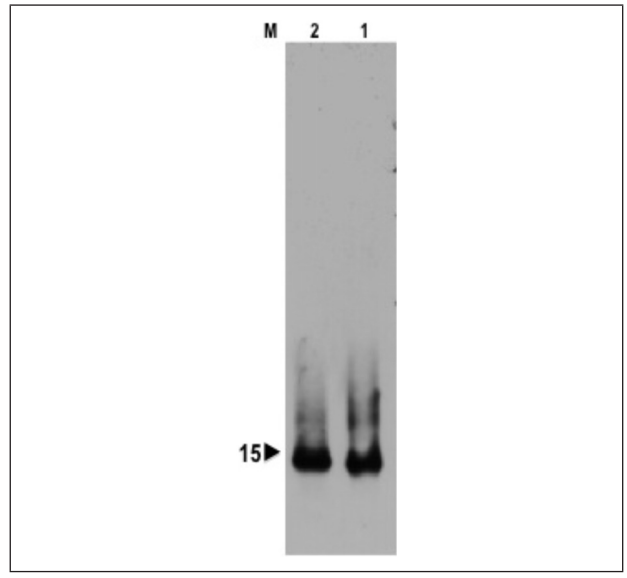

Figure 4. Immunoblot of chenopod pollen proteins probed with recombinant maize profilin 3 (ZmPRO3). Lane (1): rural pollen; lane (2): urban pollen and lane (M): molecular weight markers (kDa)

Table 1. Molecular weight (MW) range and optical density (OD mm^2) of IgE reactive protein bands of chenopod pollen extracts from urban and rural areas to weed polysensitized patients and recombinant maize profilin 3 (ZmPRO3)

Pollen extracts	Patient sera								
	MW (kDa)	A	B	C	D	E	F	G	ZmPRO3
***C. album* urban pollen**	83-80	–	–	3.06	9.42	0.58	–	1.46	–
	73-68	4.34	0.88	10.87	7.02	1.76	0.75	4.79	–
	63-61	4.02	–	1.95	–	–	0.20	2.22	–
	55-51	8.26	3.36	5.66	0.38	1.74	2.91	5.68	–
	48-40	23.83	–	6.89	–	0.41	–	2.25	–
	39-37	–	0.23	18.65	1.85	0.31	1.07	1.50	–
	35	–	–	–	–	0.36	–	2.02	–
	26	–	–	–	–	–	18.14	–	–
	14	–	–	1.16	–	–	–	–	28.91
***C. album* rural pollen**	83-80	–	–	0.80	3.87	0.40	–	2.74	–
	73-70	–	–	–	5.24	1.64	0.62	5.87	–
	69-62	1.00	–	4.20		–	–	1.31	–
	55-51	4.25	0.21	7.40	0.46	0.60	1.24	3.78	–
	48-40	–	–	2.70	–	0.08	1.04	6.16	–
	39-37	2.78	–	1.16	2.82	–	–	–	–
	35	–	–	–	–	0.30	–	–	–
	26	–	–	–	–	–	3.18	–	–
	14	–	–	0.55	–	0.58	–	–	26.56

IgE values (kU/L): 5.87 (A), 2.09 (B), 30.40 (C), 6.40 (D), 2.13 (E), 4.20 (F), 12.60 (G)

DISCUSSION

In the present study, the urban *Chenopodium album* pollen surface showed a modified structure, presenting a fine film covering its wall, deformed opercules and submicronic particles adhered to its surface previously identified as diesel exhaust, using Micro-raman spectroscopy[12]. Diesel exhaust particles have been described to promote IgE production, Th2 cytokine synthesis and to increase the permeability of local tissue to the penetration of aeroallergens[13, 14].

Several studies reported morphological pollen modifications and the presence of particles adhered to pollen wall from urban environments[15-17] compared with rural pollen. Also, *in vitro* experiments, showed that treatments with certain atmospheric pollutants can induce not only quantitative but also qualitative changes, with regard to pollen morphology and protein release[15-17]. These changes may facilitate the allergen release from pollen and the presence of adhered particulate matter may act as adjuvant or enhance the immune system, facilitating sensitisation in predisposed patients[9,18].

In our study, the total protein of pollen grains decreased in urban pollen and different protein profiles were observed in rural and urban pollen, since some protein bands seem to be absent from the urban pollen. In a previous study with *Chenopodium album* pollen extracts, SDS-PAGE protein profiles showed that in the urban pollen protein bands of 16 and 36 kDa disappeared[12]. Differences in protein profiles between pollen coming from non-polluted and polluted regions have been reported in previous studies[18-20], that showed lower soluble protein content in pollen from polluted environments. This decrease may be related to differences in protein expression between rural and urban areas[21]. Nevertheless, these differences do not seem consistent for every pollen types since Behrendt *et al.*[22,23] and Helander *et al.*[24], reported no difference between protein bands in the pollen extracts collected from non-polluted and polluted areas.

In both urban and rural pollen extracts, two reactive bands were shared by all the patients' sera tested, with approximately 70 and 53 kDa. Also, a 40 kDa band was present in five of the tested patients sera. These new groups of bands do not correspond to the chenopod allergens already characterized and purified as Che a 1 of 17 kDa [2], Che a 2 of 14 kDa and Che a 3, a polcalcin of 9.5 kDa[5]. These findings may indicate a different sensitization profiles in our patients, when compared with other studies, as well as the importance of characterizing and purifying these proteins as well as ascertaining the possible cross-reactivity with other pollen types. In fact, a band with molecular weight of 55kDa has already been detected by Wurtzen *et al.*[25] that can be analogous to the band with molecular weight around 53 kDa observed in our study. The presence in our study of two other non-characterized bands around 25 and 35 kDa has already been reported[25].

A band with similar molecular weight of Che a 1, a glycoprotein, seems to be present in patient serum F and Che a 2, a profilin, in patient sera C and E, as it was also recognized by polyclonal antibody against maize pollen profilin ZmPRO3. These results confirm that the identified band in *Chenopodium album* pollen protein extract is a profilin-like protein and indicates the presence of cross-reactivity with grasses.

Bands with similar molecular weight of Che a 3, a polcalcin-like protein, were not observed when probed with patients' sera.

In a general overview, when *Chenopodium album* pollen saline protein extracts were probed against the seven allergic patients' sera, higher optical density values were observed in the pollen extracts from the urban area. This may indicate differences in sensitization levels to the same pollen type, depending on its origin, in our case higher sensitization to urban chenopod pollen than to rural one. A recent study by Bryce *et al.*[21] showed that the different sensitization levels are possibly related to a higher chemotactic activity on human neutrophils of pollen extracts collected in urban areas. These findings are in accordance with the recent literature reporting an increase in allergy prevalence in urban environments due to atmospheric pollution.

CONCLUSIONS

Our study reported differences in *Chenopodium album* pollen morphology, protein profile and allergenicity between pollens from rural and urban origin.

Urban pollen soluble proteins showed higher reactivity with the sera of allergic patients polysensitized to wx1, indicating that urban environments with higher atmospheric pollution levels can influence airborne pollen allergenicity.

The immunological study revealed new *Chenopodium album* allergens, different from those already characterized and purified, with molecular weight of approximately 70, 53 and 40 kDa, that were common to the different sera tested and that would be interesting to study in more detail. Also, our study seems to confirm the existence of cross-reactivity between pollen allergens *of Chenopodium album* and grasses.

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Correspondence to:
Ilda Abreu ianoronh@fc.up.pt

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