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Lázaro G. Cuinica^{ab}, Ana Cruz^c, Ilda Abreu^b & Joaquim C. G. Esteves da Silva^a

^a Centro de Investigação em Química (CIQ-UP), Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Porto, Portugal

^b Faculdade de Ciências, Departamento de Biologia e Grupo do Ambiente do Centro de Geologia da, Universidade do Porto, Porto, Portugal

^c Clinical Patology Service, Immunology Laboratory, Vila Nova de Gaia Hospitalar Centre, Vila Nova de Gaia, Portugal

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Effects of atmospheric pollutants (CO, O₃, SO₂) on the allergenicity of *Betula pendula*, *Ostrya carpinifolia*, and *Carpinus betulus* pollen

Lázaro G. Cuinica^{a,b}, Ana Cruz^c, Ilda Abreu^b and Joaquim C. G. Esteves da Silva^{a*}

^aCentro de Investigação em Química (CIQ-UP), Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Porto, Portugal; ^bFaculdade de Ciências, Departamento de Biologia e Grupo do Ambiente do Centro de Geologia da, Universidade do Porto, Porto, Portugal; ^cClinical Patology Service, Immunology Laboratory, Vila Nova de Gaia Hospitalar Centre, Vila Nova de Gaia, Portugal

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Pollen of *Betula pendula*, *Ostrya carpinifolia*, and *Carpinus betulus* was exposed *in vitro* to relatively low levels of the air pollutants, namely carbon monoxide, ozone, and sulfur dioxide. The allergenicity of the exposed pollen was compared with that of non-exposed pollen samples to assess if air pollution exposition affects the allergenicity potential of pollen. The immunodetection assays indicated higher IgE recognition by all sera of allergic patients to the pollen protein extracts in all exposed samples in comparison to the non-exposed samples. These results show that the pollen exposition to low pollutants' levels induces increased allergic reaction to sensitized individuals.

Keywords: allergic sensitization; pollen allergy; *Betula pendula* pollen; *Ostrya carpinifolia* pollen; *Carpinus betulus* pollen; air pollutants

Introduction

Under contaminated environment, the pollen is exposed to levels of chemical substances that can induce alterations of its wall and biochemical properties (Ruffin et al. 1986; Bist et al. 2004; Motta et al. 2006; D'Amato et al. 2010). These interaction of pollen/pollutants may be responsible for the aggravation of the respiratory allergic diseases namely asthma and/or allergic sensitization in humans living in urban and industrial areas (Wyler et al. 2000; Gilles et al. 2009; Traidl-Hoffmann et al. 2009; Bosch-Cano et al. 2011).

The prevalence of pollinosis is increasing (about 40 %) during the last two decades, particularly in industrialized countries, but the reasons for this trend are not straightforward (Traidl-Hoffmann et al. 2009; D'Amato et al. 2010). Several environmental and epidemiological studies showed that in the European Community countries, between 8 and 35 % of the adults and youngsters have increased IgE sensitization to pollen allergens and this problem is more frequent in individuals living in urban and industrialized centers when compared with those living in rural areas (D'Amato 2000; Majd et al. 2004). These observations suggest that air pollutants present at higher levels in urban centers can modify the chemical structure of proteins present in pollen, provoking significant increases in the IgE response of exposed individuals (Motta et al. 2006;

*Corresponding author. Email: jcsilva@fc.up.pt

Cariñanos & Casares-Porcel 2011; Kim et al. 2013). Although there are significant regional differences in Europe pollinosis is usually caused by pollen from grasses, Urticaceae, Betulaceae, olive, and cypress (Filon et al. 2000; D'Amato et al. 2007, 2010; Peternel et al. 2007).

The air contaminants carbon monoxide (CO) and sulfur dioxide (SO₂) are mainly emitted to the atmosphere by the burning of fossil fuels, industrial activities, and motorized traffic (Kammerbauer et al. 1987). Ozone (O₃) is a secondary air pollutant resulting for photochemical smog reactions that begin with nitrogen oxides and volatile hydrocarbons (Karlsson et al. 2007). Taking into consideration their origin, these three air contaminants are always present in urban centers. Also, their air concentration is usually monitored in populated areas to check if the corresponding levels follow national and/or international regulations proposed for human health protection.

Betulaceae family trees belong to the list of frequent plant species used as ornamental in urban environments (Cariñanos & Casares-Porcel 2011). Although this urban tree planting is sought to promote air quality and respiratory health, besides aesthetic purposes, currently there are increasing concerns about the increasing allergenic impact on residents. Indeed, besides pollen, in city centers, the increased concentration of air pollutants is usually directly responsible for the enhancement of allergic diseases; and, the synergistic interaction of air pollutants and pollen may result in further human allergenic sensitization. Previous research on the pollen of the Betulaceae family trees has shown significant effects even with low levels of the pollutants, CO, O₃, NO₂, and SO₂ considered save for human health, on pollen fertility (Cuinica et al. 2013, 2014a, 2014b). Also, immunodetection assays indicated higher IgE recognition by patient sera sensitized to the pollen protein extracts exposed to these low levels of NO₂ in comparison to the non-exposed samples (Cuinica et al. 2014a). In the case of NO₂, the increase in the pollen allergenicity exposed to this air pollutant may be due to the nitration potential of proteins (Franze et al. 2005; Shiraiwa et al. 2012; Cuinica et al. 2014a). In the case of other air pollutants, without nitrogen and quite different chemical reactivity mechanisms, as is the case of CO, O₃, and SO₂, fewer information exist about their effect on the pollen allergenicity potential.

The objective of this work was to study the effect of low atmospheric levels of CO, O₃, and SO₂ in the allergenicity of *Betula pendula*, *Ostrya carpinifolia*, and *Carpinus betulus* pollen. In order to assess the effect of these air pollutants in the pollen allergenicity potential, the electrophoretic profiles of the protein extracts were acquired and the pollen allergenic changes were evaluated according to the binding affinity to specific IgE of sensitive patients (immunoblots).

Materials and methods

Pollen samples

B. pendula, *O. carpinifolia*, and *C. betulus* are ornamental trees that produce staminate flowers (Chen et al. 1999). Anthers were collected during the flowering season (first days of spring) in the two places where these trees exist in larger quantities (Botanic Garden and City Park of Porto) and were dried at 27 °C, gently crushed, and the pollen thus released was passed through different grades of sieves to obtain pure pollen. After, the isolated pollen was stored at −20 °C.

In vitro pollen exposure to CO, O₃, and SO₂

Pollen samples were artificially exposed in a chamber made of acrylic and wood mix with dimensions of about 50 × 70 × 50 cm³ and described in the literature (Sousa et al. 2012). The sequence of the experiments was randomized. Each pollen sample [300 mg of dry weight] was exposed individually with CO, O₃, and SO₂ under two different concentrations levels – the low level was used for one day (+) and the high level for one (++) and two days (2d++). The lower level corresponds to a concentration at about the current atmospheric hour-limit value acceptable for human health protection in Europe (8.73 ppm for CO, 0.061 ppm for O₃, and 0.13 ppm for SO₂), and the higher level, at about three times the lower level (European Union Directive 2008/50/EC of 21 May 2008 on ambient air quality and cleaner air for Europe). Pollen samples were placed into a Falcon tube with the bottom cut-off and 23 µm grids (SEFAR PET 1000) were placed on both sides – this sampling system allows the circulation of the air through it and the re-suspension of the pollen.

SO₂ (99.9 % v/v) concentrations were attained by injecting small volumes of gas directly obtained from bottles of compressed gas (Sigma–Aldrich). CO was generated by chemical reaction of sulfuric acid with formic acid – it was generated by mixing the stoichiometric amounts of the two concentrated acids in a test tube, and was subsequently injected into the chamber. Three injections of gas per assay were performed using a micro syringe through septa placed at the front chamber wall within each 2 h interval, in order to prevent greater concentration fluctuations. Ozone was generated using an A2Z OZONE system coupled to an OMRON H3DK-S1 timer, controlling the injection of ozone for brief periods of time in order to offset the high ozone reactivity. Non-exposed pollen samples were used as control. The corresponding average pollutants levels, temperature, and humidity of the experiments were previously described (Cuinica et al. 2013, 2014b).

Extraction and quantification of soluble proteins

Pollen grains (50 mg) were suspended in 1:20 (w/v) phosphate buffer saline at pH 7.4 at 4 °C. Total soluble proteins were extracted in the same buffer by continuous stirring for 4 h. The suspension was centrifuged at 13,200 rpm for 30 min at 4 °C. The supernatant was filtered through a 0.45 µm Millipore filter and centrifuged once again. The soluble protein content of all pollen extracts was quantified colorimetrically with the Coomassie Protein Assay Reagent (Pierce) by the Bradford Method (Bradford 1976).

SDS-PAGE and immunoblots

Proteins were separated in 12.5 % polyacrylamide gels (30 µg per lane) under reducing conditions (Laemmli 1970), and were visualized by Coomassie Brilliant Blue R-250 staining. The molecular weight of protein bands was estimated by comparison with an established protein marker (PageRuler Plus Prestained Protein Ladder, Fermentas). For immunoblotting analysis, the protein was electrophoretically transferred onto nitrocellulose membranes (Protran, Whatman Schleicher and Schuell, Germany). The membranes were saturated for 1 h in a blocking solution (5 % non-fat dry milk (w/v), 0.1 % goat serum (v/v), in 20 mM Tris, 150 mM NaCl (TBS), and 0.1 % Tween) and then incubated overnight at 4 °C with sensitized and non-sensitized patient sera to Betulaceae pollen proteins diluted to 1:10 in blocking solution to assess IgE-binding affinity to Betulaceae

pollen proteins. After washing, blots were probed with mouse anti-human IgE:HRP (1:2000). The reaction was revealed using peroxidase ECL substrate and exposed to ECL-Hyperfilm (GE-Healthcare). The antigenic profile bands of the SDS-PAGE and immunoblots were quantified by the software MyImageAnalysis v1.1 (Thermo Fisher Scientific Inc.).

Results and discussion

Total soluble proteins

In order to check if the pollen exposure to the three air contaminants would affect the polypeptide profiles of the pollen extracts, the corresponding electrophoretic profiles were obtained for the three pollen samples and compared with the non-exposed pollen (Figure 1). The analysis of this figure reveals no detectable difference among the exposed and non-exposed pollen samples. All the electrophoretic profiles have in common several bands ranging from 70 to 15 kDa and the most clearly defined are: 70, 55, 35, 25, 15 kDa. This result shows that the exposure of pollen to the three air contaminants does not modify significantly (within the selectivity and sensitivity of the SDS-PAGE technique) the chemical properties of the aqueous pollen extracts, i.e. the water solubility and molecular weight of the soluble proteins. These observations agree with previous results from *Betula* (Betulaceae) and *Acer negundo* pollen (Sousa et al. 2012; Cuinica et al. 2013, 2014a, 2014b). Other authors also reported no measurable differences in the polypeptide profiles of protein extracts of exposed and not exposed to air pollutants pollen (Rezanejad 2007). However, Rezanejad et al. (2003) described a significant decrease in the intensity of several bands corresponding to soluble proteins from *Lagerstroemia indica* L. pollen exposed to SO₂, NO₂, and CO for 10 and 20 days in the polluted areas of Tehran city. Similar results were described by Cortegano et al. (2004), which suggested that the exposure of pollen to air pollutants would induce structural chemical modifications in the pollen proteins. These different observations may result from different plant/pollen sensitivity to air pollutants and different concentrations of the chemical substances at which the pollen was exposed.

Immunoblots

To assess the effects of the three air pollutants on the allergenicity of the pollen samples under investigation, 24 immunoblots were performed with sera from different sensitized individuals to Betulaceae pollen protein extracts (8 for each plant) – 9 typical plots are shown in Figure 2 (3 for each plant). Allergen contents changes were evaluated according to the binding affinity to specific IgE, which results in higher optical densities (Figure 2) – the higher IgE-reaction bands (darker spots in the immunoblots) were revealed by increased optic densities (increased bars in the plot below the immunoblots).

Increased optical densities were observed in several protein bands of the pollen exposed to the three air pollutants when compared with the non-exposed pollen (Figure 2). So, after analysis of the allergen content, it was observed that the three Betulaceae pollen, when exposed to CO, O₃, and SO₂, showed several reactive bands having multiple potential allergens. The results obtained for the three pollen samples are:

- *B. pendula* pollen show that among the eight patient sera used, 100 % recognized protein bands around 58 and 17 kDa; 62.5 % around 45, 35, and 15 kDa; 37.5 % around 27 and 24 kDa; 25 % around 18 kDa; and 12.5 % around 20 kDa.

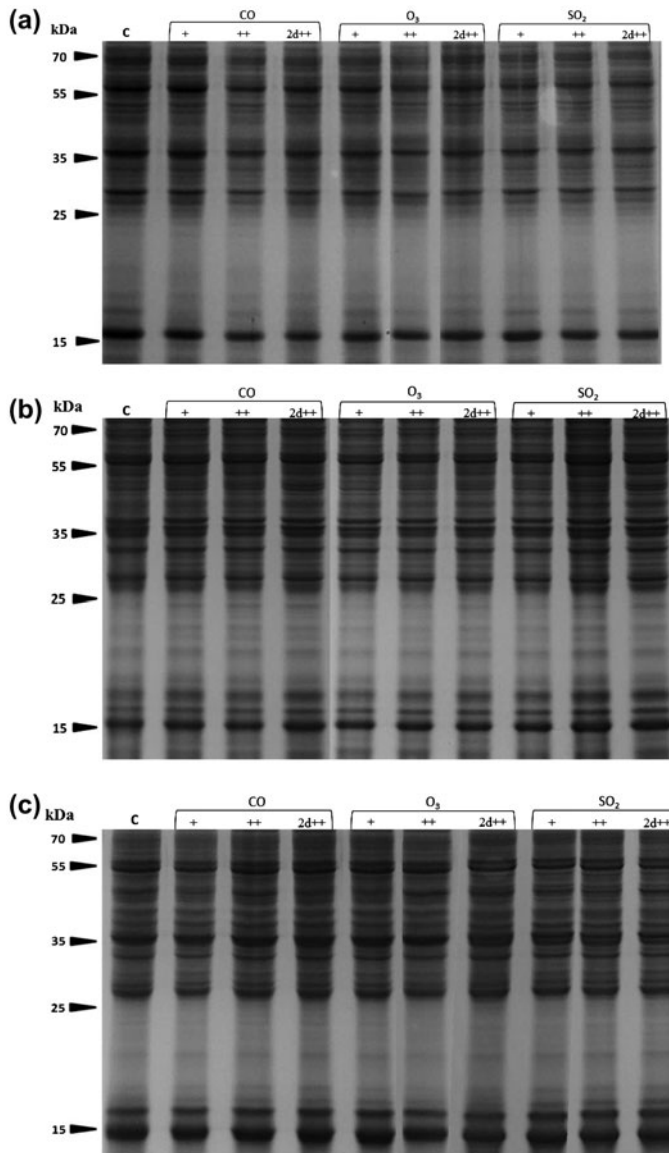


Figure 1. SDS-PAGE of soluble protein of *B. pendula* (a), *O. carpinifolia* (b), and *C. betulus* (c) pollen extracts exposed to CO, O₃, and SO₂. Signs above the lanes represent gas concentration level. C – Control; + – Low level; ++ – High level, and 2d++ – High level two days of exposure.

- *O. carpinifolia* pollen show that among the eight patient sera used, 100 % recognized protein bands around 60, 58, 35, 22, and 17 kDa; 87.5 % around 29, 27, 24, and 15 kDa; and 37.5 % around 42 and 18 kDa.
- *C. betulus* pollen show that among the eight patient sera used, 100 % recognized protein bands around 58, 45, 35, and 31 kDa; 87.5 % around 60 kDa; 62.5 % around 29, 24, and 17 kDa, 37.5 % around 42, 25, 22, and 20 kDa; and 12.5 % around 53 kDa.

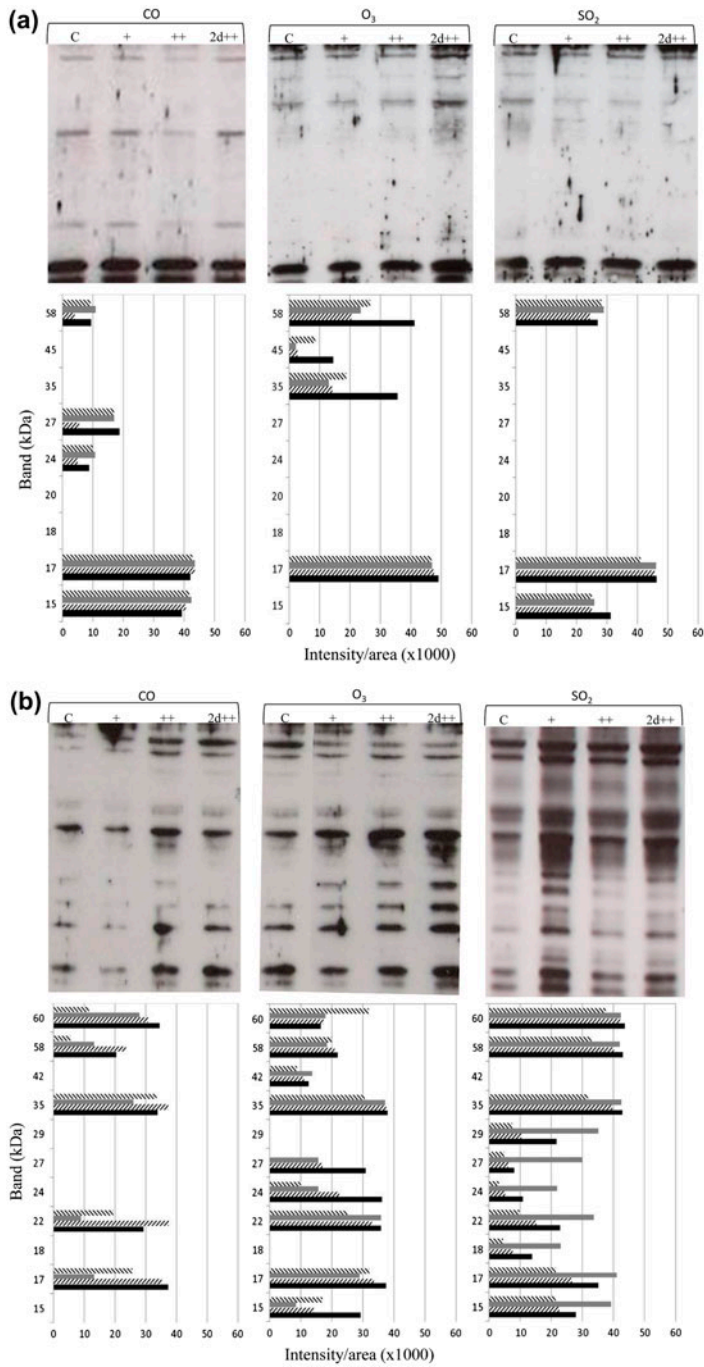


Figure 2. Immunoblots and optic densities (O.D.) of IgE-reactive bands of *B. pendula* (a), *O. carpinifolia* (b), and *C. betulus* (c) pollen extracts exposed to CO, O₃, and SO₂. Signs above the lanes represent gas concentration level: C – Control; + – Low level; ++ – High level, and 2d++ – High level two days of exposure.

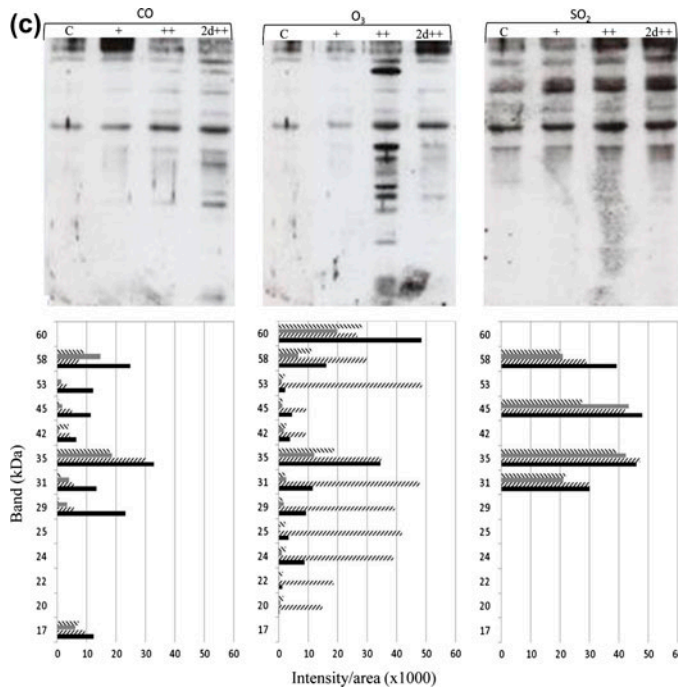


Figure 2. (Continued).

The analysis of these results allows the detection of two reactive bands common to the three plants, namely at 58 and 17 kDa, which were also detected when these pollen samples were exposed to NO₂ (Cuinica et al. 2014a), suggests a non-specific reaction to one particular air pollutant. Accordingly to Patriarca et al. (2000), a 17 kDa protein is the main allergen present in the Betulaceae pollen and its reactivity towards IgE is due to the presence of Bet v1. But, the allergen, with a molecular mass of 58 kDa, recognized by all the assayed sera, was not yet characterized and identified in the Betulaceae family pollen. Following the official website for the systematic allergen nomenclature (<http://www.allergen.org/>), allergens corresponding to 35, 24, 18, 17, and 15 kDa may correspond to antibodies already characterized and designed by Bet v6, Bet v3, Bet v7, Bet v1, and Bet v2, respectively (Cuinica et al. 2014a).

The specific effect of the air pollutant

The analysis of the set of immunoblots obtained for each plant (Figure 2), taking into consideration the pollutant level and time of exposure, shows some particularities:

- In the *B. pendula* pollen, the IgE reactivity was higher in allergens present in the pollen exposed to the higher pollutant level for two days for all the three pollutants.
- In the *O. carpinifolia* pollen, the IgE reactivity was higher in allergens present in the pollen exposed to the higher pollutant level for two days for O₃ and SO₂ and to the higher pollutant level for both exposure times for CO.

- In the *C. betulus* pollen, the IgE reactivity was higher in allergens present in the pollen exposed to the higher pollutant level for two days for CO and SO₂ and to the higher pollutant level for both exposure times for O₃.

These results demonstrate that the pollen exposure to the three air pollutants under investigation increase the allergenicity and may induce higher IgE reactivity in sensitive patients to the pollen of these plant species. Similar results were observed by Sousa et al. (2012) that reported high IgE reactivity to allergens in *A. negundo* pollen after *in vitro* exposure to SO₂ and NO₂. Other authors (Behrendt et al. 1997) showed that SO₂ and NO₂ increase the allergenic potential of pollen. Air pollutants cause stress in plants, inducing the regulation of several protein correlated with pathogeneses and defense system as reported for *Cupressus arizonica* pollen exposed to the polluted atmosphere at Toledo (Spain) (Cortegano et al. 2004). The similarity of the present results, obtained after exposure to three air pollutants that contain no nitrogen, with those obtained with NO₂ for the same plants under investigation (Cuinica et al. 2014a), confirms that a non-specific pollutant effect is observed in the increased allergenicity of pollen.

This study shows that the pollen of *B. pendula*, *O. carpinifolia*, and *C. betulus* contains several proteins potentially allergenic that can be modified by chemical substances present in the atmosphere. Bowler and Crapo (2002) suggested that O₃ can modify the chemical structure of the proteins present in pollen through the generation of reactive oxygen species, which can increase the allergenicity. NO₂ can increase the allergenic potential by the direct nitration of allergens (Franze et al. 2005; Gruijthuijsen et al. 2006). SO₂ and CO can affect the morphology of pollen and induce an increase in free aminoacids which may be responsible for an increase in the allergic symptoms in exposed individuals (Ruffin et al. 1986; Bist et al. 2004).

Conclusions

The exposure of pollen from *B. pendula*, *O. carpinifolia*, and *C. betulus* to air concentrations of CO, O₃, and SO₂ close to the current atmospheric hour-limit value acceptable for human health protection in Europe induces an increase in the pollen allergenicity, at least to atopic individuals. The allergens common to the three plants correspond to proteins with 58 and 17 kDa. Further research is needed for the characterization of the allergen of 58 kDa to assess the allergenic potential and to develop new immunoassays about Betulaceae pollen allergenicity. Future work will also focus on the difference between the intact and fragmented pollen to check if air pollutants react only with the surface and/or inside proteins.

The concentration levels of the air pollutants used in this work were around those considered safe for human health. However, as demonstrated, those levels produced measurable immunological responses which would affect human health, at least of those with allergic diseases. Consequently, further studies are necessary about the currently atmospheric limits considered safe for humans. This discussion should include the synergistic effects that results from the interaction of air pollutants and bioaerosols, such as the increase in allergic diseases prevalence.

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