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Changes in the IgE-reacting protein profiles of *Acer negundo*, *Platanus x acerifolia* and *Quercus robur* pollen in response to ozone treatment

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This study aims to investigate the effects of O₃ in protein content and immunoglobulin E (IgE)-binding profiles of *Acer negundo*, *Platanus x acerifolia* and *Quercus robur* pollen. Pollen was exposed to O₃ in an environmental chamber, at half, equal and four times the limit value for the human health protection in Europe. Pollen total soluble protein was determined with Coomassie Protein Assay Reagent, and the antigenic and allergenic properties were investigated by SDS-PAGE and immunological techniques using patients' sera. O₃ exposure affected total soluble protein content and some protein species within the SDS-PAGE protein profiles. Most of the sera revealed increased IgE reactivity to proteins of *A. negundo* and *Q. robur* pollen exposed to the pollutant compared with the non-exposed one, while the opposite was observed in *P. x acerifolia* pollen. So, the modifications seem to be species dependent, but do not necessarily imply that increase allergenicity would occur in atopic individuals.

Keywords: ozone; pollen; IgE; soluble proteins; allergenicity

Introduction

Over the past years, an increase in prevalence and severity of respiratory allergic diseases related with atmospheric pollen has been observed, particularly in industrialized countries. This is considered a worldwide problem with serious implications on the economy, related to the decrease in workers' productivity or to the absence from work due to appearance or exacerbation of allergic respiratory symptoms such as rhinitis, conjunctivitis, asthma and occasionally urticaria. Also, these diseases present a high economic burden on the healthcare system due to high resource allocations to treatment and preventive management programmes.

Type I hypersensitive reactions, such as allergic rhinitis and allergic asthma, are based on the production of immunoglobulin E (IgE) antibodies against allergens (for instance, proteins present on pollen grains) (Traidl-Hoffmann et al. 2009; Rouvinen

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et al. 2010). Usually, these pollen allergenic proteins have specific functions, as in pollen germination or in pollen–stigma interactions.

The increase in respiratory allergies has been usually related with environmental exposure and includes many diverse causes related with air quality (Ring 2012). This phenomenon has been linked to the interaction between pollen allergens and gaseous pollutants such O₃, SO₂, NO₂, or CO (D'Amato 2011; Gasana et al. 2012). One hypothesis proposed to explain this increase is based on the fact that air pollutants, such as O₃, may alter pollen biochemical characteristics, increasing its allergenic potential, thus leading to an aggravation of allergic symptoms in susceptible individuals or development of symptoms in non-symptomatic ones (Rogerieux et al. 2007; Rezanejad 2009; Traidl-Hoffmann et al. 2009; Bosch-Cano et al. 2011; Sousa et al. 2011, 2012; Gasana et al. 2012).

Tropospheric ozone is currently accepted as one of the most important atmospheric pollutant for the twenty-first century, and the increase in its concentration is a problem of international dimension (WHO 2000). At ground level, ozone is mainly formed by photochemical reactions, with nitrogen oxides (NOX), carbon monoxide (CO) and volatile organic compounds as precursors.

Ozone induces short-term acute effects such as respiratory symptoms, pulmonary function changes, increased airway responsiveness and airway inflammation. These responses occur at or close to the upper limits of background concentrations (WHO 2000). Background levels of ozone are usually in the 40–70 µg/m³ range (0.020–0.035 ppm). However, maximum hourly ozone values in Europe have exceeded 350 µg/m³ (0.180 ppm) in urbanized regions (WHO 2000).

The European Union fixed safety standards for O₃ that should not be exceeded. According to the Directive 2008/50/EC, (i) information threshold – 180 µg/m³ (0.085 ppm; 1-h average); (ii) alert threshold – 240 µg/m³ (0.114 ppm; 1-h average); and (iii) limit value for the protection of human health – 120 µg/m³ (0.061 ppm; daily maximum 8-h average threshold, not to be exceeded more than 25 days per calendar year, determined as a three-year average starting from 2010).

The interaction degree between air pollutants, airborne pollen allergenicity and the immune system is still marginally understood with many uncertainties. To better understand the complex relationship between the environment and pollinosis, it would be interesting to see if the interaction between airborne pollen and gaseous pollutants is dependent on plant species and/or gas concentrations. So, the aim of this study was to investigate the effects of O₃ on protein content and IgE-binding protein profiles of *Acer negundo*, *Platanus x acerifolia* and *Quercus robur* pollen after *in vitro* exposure to different concentration levels of the gas. These tree species are ornamental plants widely planted throughout the Porto city, not only in public parks and gardens as in sidewalks. They present also overlapping pollination seasons that contribute to an exacerbation of respiratory allergic symptoms.

Materials and methods

Pollen samples

A. negundo, *P. x acerifolia* and *Q. robur* anthers were collected in the city of Porto, northwest of Portugal, in public gardens and sidewalks, during the flowering period. The trees selected were easy to access, free standing, with abundant branches and presented flowers with dehiscent anthers. In each tree, the inflorescences were randomly

collected from all quadrants in different branches until a 30×10 cm box was filled. 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 stored at -20°C .

In vitro exposure to ozone

Pollen samples were exposed to O_3 in an environmental chamber equipped with a Solar Simulator (Newport Oriel 96000 150 W), a fan (SUNON SF23080AF) to homogenize the air and temperature and relative humidity sensors (EBRO EBI20 sensor) (Sousa et al. 2012). The reproducibility of exposure conditions was demonstrated in Ribeiro et al. (2013).

Pollen samples of 250 mg of dry weight (DW) were placed in a tube with both edges closed by a $23\text{-}\mu\text{m}$ pore length mesh (SEFAR PET 1000). This tube was then placed over a fan that impelled the air within the chamber to pass through the tube. Pollen was exposed to O_3 during 6 h at three different concentrations, chosen based on the European Union Directive 2008/50/EC of 21 May 2008 on ambient air quality and cleaner air for Europe. Pollen samples were individually exposed to O_3 at approximately half (+), equal (++) and four times (+++) the limit value for the protection of human health in Europe (0.061 ppm; daily maximum 8-h average threshold) (Table 1).

O_3 concentrations were attained using an ozone generator (A2ZS-1GLAB) and continuously monitored using an O_3 concentration sensor (AEROQUAL Series 500 sensors) 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. A non-exposed pollen sample was used as control.

Protein extraction and quantification

Pollen grains (50 mg DW) were suspended in 1:20 (w/v) phosphate buffer saline at pH 7.4 with continuous stirring for 4 h at 4°C . The suspension was then centrifuged at 16,100 g for 30 min at 4°C . The supernatant was filtered through a $0.45\text{-}\mu\text{m}$ 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 (1976). A standard curve estimated using different BSA concentrations (0–2000 $\mu\text{g/ml}$) was used as a protein standard. Three replicate measurements per extraction were performed, and a Student's *t*-test was used to evaluate the significance of quantitative changes in soluble protein content after exposure to O_3 , for each pollen species, compared to the control sample. Values of $p < 0.05$ were considered significant (Table 1). The reproducibility of protein extraction and quantification methods was demonstrated in Ribeiro et al. (2013).

SDS-PAGE and immunoblots

Proteins from pollen extracts were separated in 12.5 % (w/v) polyacrylamide gels under reducing conditions (20 μg per lane), and the proteins 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 bands separated by SDS-PAGE (20 μg per lane) were electrophoretically transferred (TE22 Mighty small

Table 1. Average and standard deviation in pollen protein content of *A. negundo*, *P. x acerifolia* and *Q. robur* exposed to different levels of O₃. *t*-Test *p*-values for differences in average pollen protein content between control and each exposure level.

<i>Acer negundo</i>			<i>Platanus x acerifolia</i>			<i>Quercus robur</i>		
O ₃ level	Protein (µg/ml)	<i>t</i> -Test (<i>p</i> -value)	O ₃ level	Protein (µg/ml)	<i>t</i> -Test (<i>p</i> -value)	O ₃ level	Protein (µg/ml)	<i>t</i> -Test (<i>p</i> -value)
C	5783.3 ± 522		C	4380.4 ± 279		C	4575.6 ± 322	
+ (0.030)	5352.2 ± 131	0.199	+ (0.029)	2949.9 ± 138	0.000	+ (0.031)	4289.6 ± 87	0.042
++ (0.058)	5834.7 ± 51	0.872	++ (0.060)	3125.4 ± 217	0.000	++ (0.058)	4185.8 ± 120	0.011
+++ (0.227)	5129.8 ± 143	0.007	+++ (0.227)	3047.1 ± 328	0.000	+++ (0.235)	4092.8 ± 131	0.005

Notes: C: control experiment; +: half the 8 h-limit value; ++: 8 h-limit value; +++: four times the 8 h-limit value for human health protection according to the European Union Directive 2008/50/EC of 21 May 2008. *p*-Values lower than 0.05 indicate the rejection of the null hypothesis at the 95% confidence level (italic values).

Transphor unit – GE Healthcare) to nitrocellulose membranes (Protran, Whatman® Schleicher & Schuell, Germany). Transfers were carried out in a solution of 192 mM glycine, 25 mM Tris and 20 % methanol (transfer buffer) during 2.5 h at 200 mA. The membranes were saturated during 1 h in a blocking solution (5 % non-fat dry milk and 0.1 % goat serum in TBS-T (20 mM Tris, 150 mM NaCl with 0.1 % Tween-20)) and then incubated overnight at 4 °C with atopic patient sera diluted 1:10 in blocking solution for identification of allergens. After washing, bound-specific IgE was detected by horseradish peroxidase-conjugated anti-human IgE serum (Southern Biotechnology Associates). An ECL solution (Luminata Crescendo, Interface, Lda.) was used as a detection system. The chemiluminescent signal was exposed to AGFA medical X-ray film and developed by Fuji medical film processor model FPM 100A. The antigenic profile bands of the SDS-PAGE and immunoblots were quantified by a Molecular Imager GS800 calibrated densitometer and Quantity One 1-D Analysis, v4.6 (Bio-Rad laboratories).

For each tree, 10 anonymous random atopic patients sera previously selected as polysensitized to *A. negundo*, *P. x acerifolia* and *Q. robur* pollen assessed by positive Skin prick test response were chosen. Each serum was used individually in the immunoblots experiments. Sera were separated from the whole blood, collected by venipuncture and allergen-specific IgE levels were measured by ImmunoCAP™ FEIA test (Phadia AB). The sera were chosen having specific IgE values higher than 0.35 kU/L. Pollen allergenic changes were evaluated according to the binding affinity to specific IgE of atopic patient sera to pollen extracts. Higher binding affinities indicate higher IgE reactivity, revealing more conspicuous bands with higher optic densities. Each serum represents only one polysensitized individual with a regular volume of around 0.5–0.7 ml, which supplies one single experiment.

Results

Total soluble protein

Differences were observed in pollen total soluble protein content of *A. negundo*, *P. x acerifolia* and *Q. robur* after exposure to O₃ (Table 1). Control samples presented significantly higher total soluble protein content compared to all exposed samples but one. The O₃ treatment induced significant decrease in pollen protein release of *P. x acerifolia* and *Q. robur*, regardless of the gas concentrations tested (half, limit and above the limit for human health protection). In *A. negundo* pollen, no significant differences were observed in protein content when pollen was exposed to half and the 8-h limit concentration for human health protection.

SDS-PAGE and immunoblots

Figure 1 shows the resolved protein profiles of the *A. negundo*, *P. x acerifolia* and *Q. robur* pollen extracts analysed after SDS-PAGE and Coomassie staining. A similar pattern for the major protein species was observed, but quantitative differences were observed between the control samples and the exposed ones. Proteins within the range of 58–52, 44 and 34–32 kDa for *A. negundo* profile and of 58–52, 47–40, 34–32 and 23–20 kDa for *Q. robur* were relatively more abundant in extracts corresponding to the exposed pollen samples when compared with the control one. On *P. x acerifolia* profile, the most conspicuous differences were those within the range of 44 and 34–32 kDa, being relatively less abundant in the extracts corresponding to the exposed pollen.

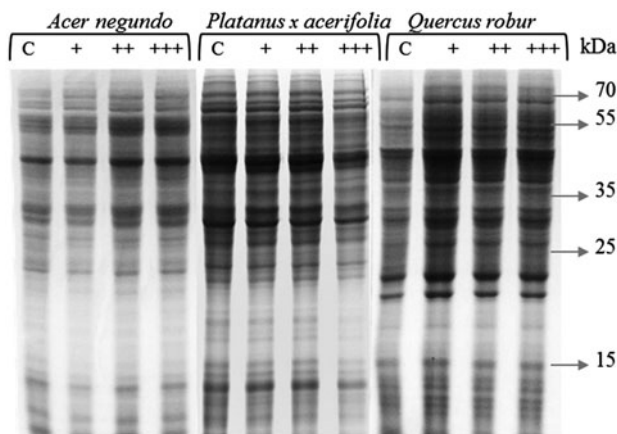


Figure 1. SDS-PAGE of total soluble proteins of *A. negundo*, *P. x acerifolia* and *Q. robur* pollen extracts exposed to O_3 . Signs above the lanes represent gas concentration level: C: control experiment; +: half the 8-h limit value; ++: 8-h limit value; +++: four times the 8-h limit value for human health protection according to the European Union Directive 2008/50/EC of 21 May 2008.

Four allergograms, corresponding to four individual sera presenting different specific IgE levels (higher than 0.35 kU/L) to *A. negundo*, *P. x acerifolia* and *Q. robur* pollen, are shown, respectively, in Figures 2, 3 and 4. Each figure represents 4 immunoblots taken as representative of the overall 10 tested. Also, the corresponding optic densities (expressed in Intensity/area) of the most conspicuous IgE-reactive bands are presented for control and exposed pollen extracts. The IgE-binding pattern of the atopic patients' sera to the control and exposed pollen extracts revealed differences regarding the number and intensity of IgE-binding proteins (Figures 2–4).

Prominent groups of IgE-reactive bands were common to the different pollen type extracts: Proteins with molecular weight of approximately 52, 43, 30, 27, 21 and 14 kDa shared by *A. negundo*, *P. x acerifolia* and *Q. robur*; of 53, 36 and 16 kDa shared by *A. negundo* and *P. x acerifolia* and finally proteins with a molecular weight of approximately 61, 38, 27, 18 and 15 kDa shared by *P. x acerifolia* and *Q. robur*.

In the *A. negundo* experiments, when compared exposed with non-exposed pollen, an increase in IgE reactivity was observed in 7 out of the 10 tested sera from sensitive individuals to this pollen type. On the other three sera, no differences were observed regardless of the O_3 exposure level. Also, some protein species were recognized by patients' sera only in O_3 exposed samples (e.g. in serum 1 a protein band of approximately 55 kDa, in serum 3 of 64 and 61 kDa) (Figure 2).

In the *P. x acerifolia* experiments, the pollen extracts corresponding to non-exposed pollen led to higher IgE reactivity in 9 of the 10 sera tested, whereas only one serum showed an increase in IgE reactivity with increasing O_3 concentrations. Also, on the majority of the tested sera, a decrease in IgE reactivity was observed with increasing O_3 concentration exposure (Figure 3).

Finally, in the *Q. robur* experiments, an increase in IgE reactivity was observed in exposed pollen compared with non-exposed pollen in 7 of the 10 tested sera from sensitive individuals to this pollen type. However, on 2 of the 10 tested sera no differences

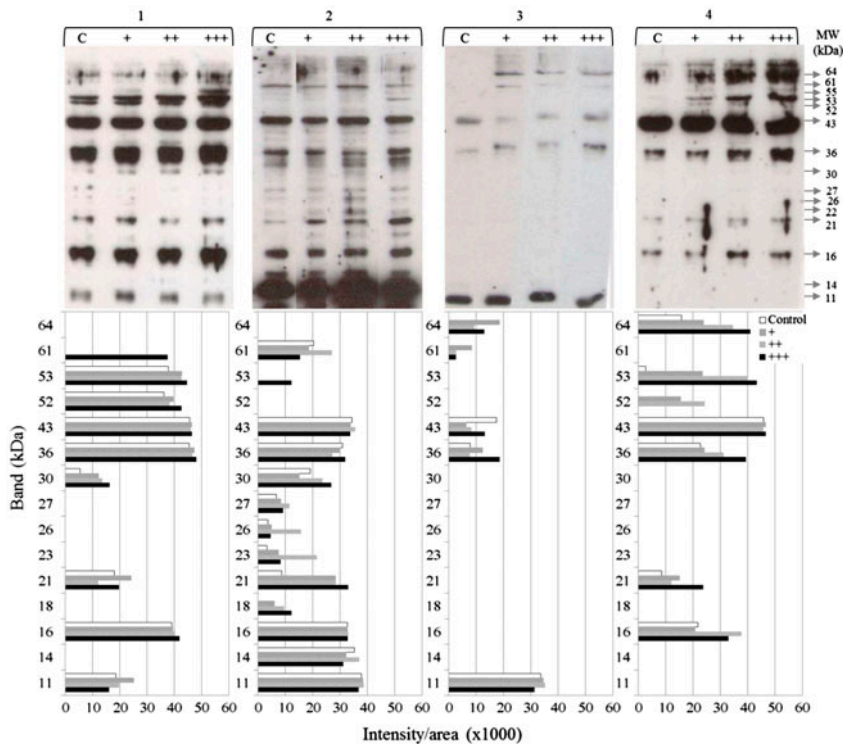


Figure 2. Immunodetection of *A. negundo* pollen extracts assayed with a panel of sera from different sensitized individuals to *A. negundo*. These 4 immunoblots were taken as representative of the overall 10. Signs above the lanes represent O_3 concentration level: C: control experiment; +: half the 8-h limit value; ++: 8-h limit value; +++: four times the 8-h limit value for human health protection according to the European Union Directive 2008/50/EC of 21 May 2008. The graphic below each immunoblot correspond to the optic densities of most prominent IgE-reactive bands observed at each O_3 concentration level. Molecular weights of bands are expressed in kDa.

in IgE reactivity were observed, and only one serum presented decreased IgE reactivity after O_3 exposure (Figure 4).

Discussion

In our study, it was observed a decrease in total soluble protein of *A. negundo*, *P. x acerifolia* and *Q. robur* pollen after O_3 exposure. Some studies have been conducted to ascertain the effects of pollution on the pollen soluble protein content. Several plant species have been studied under *in vivo* or *in vitro* conditions of exposure to pollutants and a decrease, increase or even no changes in pollen total soluble protein have been reported (Majd et al. 2004; Rezanejad 2009; Pasqualini et al. 2011; Sousa et al. 2011, 2012). These different trends are possibly related to the dissimilarities in the experiment design of these studies, with differences in plant species studied and exposure conditions, and therefore cannot be comparable. However, taken together, these results point out that the effects of atmospheric pollutants on pollen protein content is most likely dependent on species and pollutant type and their concentration. In fact in our study, the response to O_3 exposure differed between the plants tested, concerning

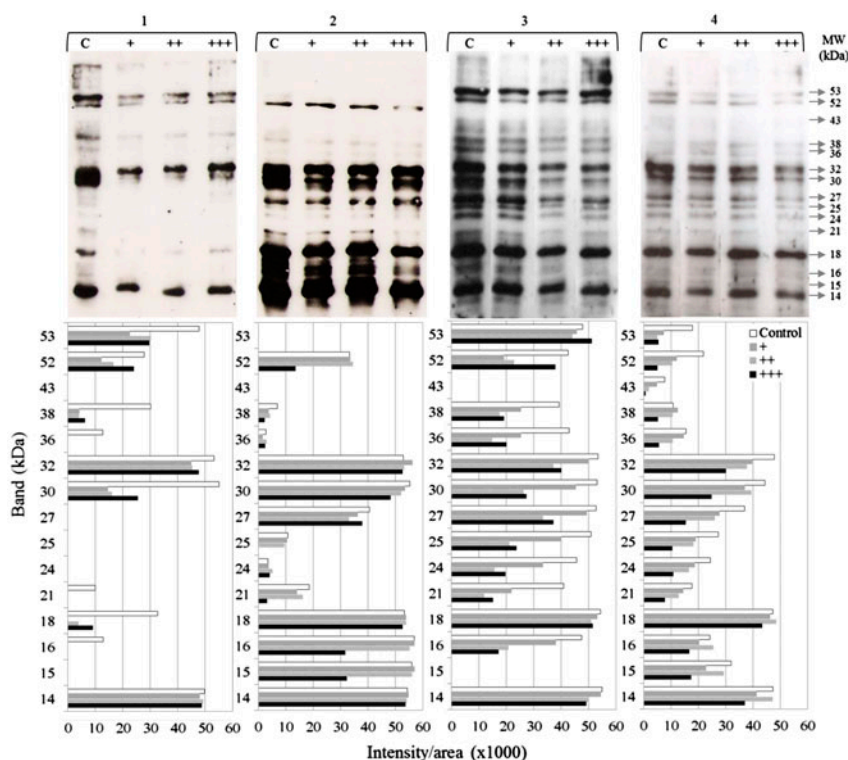


Figure 3. Immunodetection of *P. x acerifolia* pollen extracts assayed with a panel of sera from different sensitized individuals to *P. x acerifolia*. These four immunoblots were taken as representative of the overall 10. Signs above the lanes represent O_3 concentration level: C: control experiment; +: half the 8-h limit value; ++: 8-h limit value; +++: four times the 8-h limit value for human health protection according to the European Union Directive 2008/50/EC of 21 May 2008. The graphic below each immunoblot correspond to the optic densities of most prominent IgE-reactive bands observed at each O_3 concentration level. Molecular weights of bands are expressed in kDa.

not only pollen soluble protein content and their protein electrophoretic profile but also the affinity to specific IgE of atopic patient sera to pollen extracts.

The protein electrophoretic profile, within each species, was similar between non-exposed and exposed pollen samples, indicating no loss of protein bands after O_3 exposure. However, protein profile quantitative differences were detected and did not present the same trend for the three studied trees. In *A. negundo* and *Q. robur* pollen, some protein species were relatively more abundant in the extracts corresponding to the exposed pollen when compared with the control one, but in *P. x acerifolia* the opposite was observed with some protein bands being relatively less abundant in the extracts corresponding to the exposed pollen. As in the pollen total protein content, different results have been reported in the literature concerning the comparison of the electrophoretic protein pollen profile between non-polluted and polluted pollen. Some studies describe no changes in the electrophoretic profile between non-polluted and polluted pollen (Rezanejad 2009); others report the disappearance of protein bands, for example, in *Canna indica* pollen exposure to a polluted area of Tehran (Majd et al. 2004) and, as

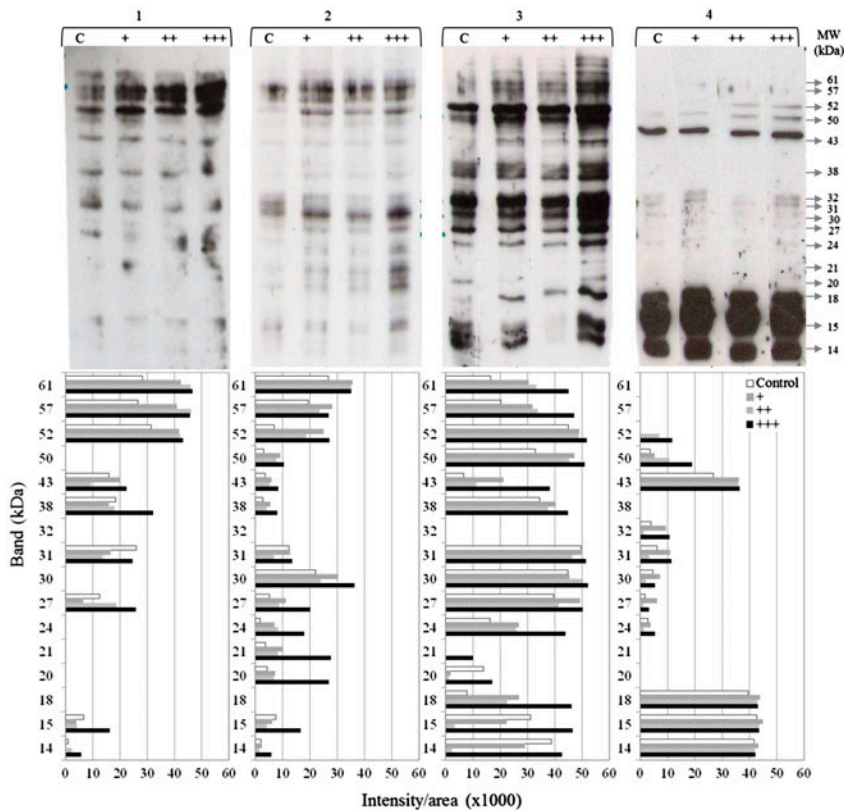


Figure 4. Immunodetection of *Q. robur* pollen extracts assayed with a panel of sera from different sensitized individuals to *Q. robur*. These four immunoblots were taken as representative of the overall 10. Signs above the lanes represent O_3 concentration level: C: control experiment; +: half the 8-h limit value; ++: 8-h limit value; +++: four times the 8-h limit value for human health protection according to the European Union Directive 2008/50/EC of 21 May 2008. The graphic below each immunoblot correspond to the optic densities of most prominent IgE-reactive bands observed at each O_3 concentration level. Molecular weights of bands are expressed in kDa.

in our study, were observed changes in the size of some bands. These quantitative changes point either towards an increase or a decrease in protein. In the present work, at reproducible exposure conditions (Ribeiro et al. 2013), both situations were observed depending on the plant tested. Therefore, these results confirm a dependency of the species on the effects of pollutants on pollen, explaining the contradictory results reported in the literature. Furthermore, the differences in protein profile were somewhat reflected in the immunoblots assayed using different patient sera sensitized to *A. negundo*, *P. x acerifolia* and *Q. robur* pollen extracts.

In the probed immunoblots, some prominent groups of IgE-reactive bands were common to the different pollen type extracts. Some of these reactive bands may correspond to already identified allergens. So far, four *Platanus* pollen allergens were identified: Pla a 1 (18 kDa non-glycosylated protein) (Asturias et al. 2002) and Pla a 2 (43 kDa) (Ibarrola et al. 2004), reported as a major allergen, Pla a 3 (a lipid transfer protein; 10 kDa) (Lauer et al. 2007) and a *Platanus* profilin (14 kDa) (Enrique et al. 2004). Furthermore, Asero

et al. (2012) reported a marked IgE reactivity at about 50 kDa on an immunoblot of a patient's serum monosensitized to plane pollen. In *Quercus*, three allergens were characterized: Que a 1 (17 kDa, a group 1 Fagales protein) (Ipsen & Hansen 1991; Niederberger et al. 1998; Movérare et al. 2008; Wallner et al. 2009); Que a CBP, a calcium-binding protein (Wopfner et al. 2007); and Que a Profilin (14 kDa) (Niederberger et al. 1998). For *Acer*, so far to our knowledge, its pollen immunological profile has been reported (Ribeiro et al. 2009), but no identification and characterization of its allergens has been performed. These common bands could indicate the existence of cross-reactivity among pollens since allergic cross-reactivity to tree pollen from various plants has been reported (Mothes & Valenta 2004). In fact, this phenomenon is connected with the ability of IgE antibodies originally raised against a given allergen to bind homologous molecules originating from a different allergen source (Hauser et al. 2010). Supporting this idea, it is the infrequent existence of mono-sensitized individuals to a single pollen type, being the majority of patients allergic to multiple allergen sources (Hauser et al. 2010).

Exposure of pollen to pollutants has been suggested to alter their allergenicity by a process of post-translational modification (PTM). Ozone can induce oxidative post-translational modification by reacting with organic surfaces that contain reactive oxygen atoms or groups, in this case with pollen grains, resulting in the formation of reactive oxygen intermediates (Shiraiwa et al. 2011; Shiraiwa, Selzle, Yang, et al. 2012). These PTMs can change the proteins function resulting in gain, inhibition or no change in function (Shiraiwa, Selzle, & Poschl 2012) that can lead to enhanced or decreased allergenicity. In our study, the O₃ effects differed depending on the plant tested. In *A. negundo* and *Q. robur*, an increase in IgE reactivity (in the majority of the tested sera) or no differences were observed in O₃-exposed samples compared with non-exposed pollen, while in *P. x acerifolia*, non-exposed pollen lead to higher IgE reactivity compared with the exposed samples. This differential behaviour has been reported in the literature. Some studies reported the presence of higher Lol p 5 or Cup a 3 in pollen collected in areas of heavy air pollution compared with pollen from less-polluted areas (Armentia et al. 2002; Cortegano et al. 2004), and García-Gallardo et al. (2013) showed an increase in allergenic potency (both on skin prick test and IgE, expression of allergenic proteins) of pine pollen recovered from areas with higher exposure to O₃. On the contrary, Rogerieux et al. (2007) found that O₃ induced a decrease in allergen detection in timothy grass pollen that could be due to a mechanical loss of allergens from the altered pollen grains and/or PTMs affecting allergen recognition by IgE. All these results confirm that the outcome of the interaction between pollen and O₃ is species dependent. Also, Kanter et al. (2013) after exposing *Ambrosia* plants to elevated ozone over the entire vegetation period reported no significant changes on the Amb a 1 pollen allergen content tested by ELISA. However, these authors point out that pollen from some plant species grown in urban habitats showed higher allergenicity even though no changes in allergen content were observed.

The way specific pollen allergens of the tested plants in our study are modified by O₃ is still to be investigated. However, these variations can be related to dissimilarities in allergen content and releasability that seem to be dependent on plant family (Gunawan et al. 2008; Golebski et al. 2013). Several pollen allergens have been identified as cysteine, serine or aspartic proteases (Gunawan et al. 2008; Golebski et al. 2013). Gunawan et al. (2008) showed that pollen of Cupressaceae/Taxodiaceae contained serine protease while birch and ragweed pollen contained both serine and cysteine protease with different degrees of releasability. These authors point to differences in the pollen structure as an explanation for these variances. Furthermore, Vinhas et al. (2011) showed that different pollen types, such as *Olea europaea*, *Dactylis glomerata*, *Cupressus sempervirens* and

Pinus sylvestris, have distinct abilities to release their soluble components, such as proteases that might be involved in the sensitization to a range of airborne allergens. So, differences in the content of the amino acids between *A. negundo*, *P. x acerifolia* and *Q. robur* and their susceptibility to oxidation by O₃ may explain the different results observed on immunoblot experiments with *P. x acerifolia*.

In general, protein oxidation processes may produce conformational variations within the protein or quaternary structural changes (dimerization or oligomerization) that lead to protein processing and presentation changes that might have a strong influence on immunogenicity and enhancement of the immune responses (Stadtman & Levine 2000, 2003; Rouvinen et al. 2010; Grimm et al. 2012; Karle et al. 2012; Shiraiwa, Selzle, & Poschl 2012). Amino acids such as cysteine, tryptophan, tyrosine and phenylalanine are oxidized by O₃, causing denaturation of the proteins by introducing changes in their secondary and tertiary structure (Cataldo 2003). Oligomerization can increase the allergenicity of a protein, as observed in Bet v 1 dimer that induced a positive skin test in allergic mice, whereas the monomer gave negative results (Scholl et al. 2005; Rouvinen et al. 2010). Having in mind that many pollen allergens have the ability to dimerization or oligomerization, particularly to form transient dimers, this can explain why in our results some proteins were recognized by the patient's sera only in O₃ exposed samples.

Conclusions

Our results demonstrated that *A. negundo*, *P. x acerifolia* and *Q. robur* pollen contains multiple potentially allergenic proteins, which can be modified by their interaction with O₃. The modifications seem to be species dependent. Also, the lowest level of O₃ tested in our study corresponds to values that are half the limit value for the human health protection, inducing changes in protein content and IgE reactivity on pollen. Whether these changes might result in an exacerbation of allergies needs to be proven by a more detailed study such as the use of ELISA or mass spectroscopy techniques to ascertain significant modifications or performing provocation tests with pollen extracts after exposure to pollutants.

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