

Effects of ozone in *Plantago lanceolata* and *Salix atrocinerea* pollen

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Abstract Pollen can be modified by the increasing environmental pollution which can lead to a rising trend in pollen-induced respiratory allergies. The aim of this work was to study the fertility, protein profile and allergenicity of *Plantago lanceolata* and *Salix atrocinerea* pollen after exposure to ozone. The pollen collected directly from the anthers of both species was exposed to O₃ in an environmental chamber, for 6 h at different concentrations. After exposing the pollen, viability tests and in vitro germination were performed. Total protein content was determined by the Bradford method, the polypeptides were separated by SDS-PAGE and the antigenic properties were analyzed by immunological techniques using patients' sera. The concentrations of O₃ tested affected the

pollen fertility and the total protein content, but the protein profiles did not show any differences. Furthermore, the immunological tests revealed an increase in IgE binding reactivity when pollen was exposed to the pollutant.

Keywords Airborne pollen · Allergenic sensitization · Ozone · Pollen allergy

1 Introduction

Pollen grains are biological structures produced as part of the reproductive cycle of the higher plants. As a consequence of the pollination process of anemophilous plants, large amount of pollen is released into the atmosphere, becoming an atmospheric allergenic responsible for respiratory problems. Also, while airborne, pollen is subjected to interactions with other atmospheric constituents that can stress both the pollinating plant and the pollen grain (Sofiev et al. 2013). Indeed, in city centers, besides the airborne pollen, the increased concentration of air pollutants is commonly directly correlated with the enhancement of respiratory diseases, and the interaction between both may result in further human allergenic sensitization (D'Amato et al. 2007).

In the past years, the interaction between airborne pollen and gaseous pollutants, such as O₃, has been studied in order to access the qualitative and

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quantitative changes in pollen features (Chichiricco and Picozzi 2007; Pasqualini et al. 2011; Ribeiro et al. 2014). These interactions with pollen cause changes on its fertility affecting the reproductive cycle of seed plants (Omasa 2002). At cellular level, air pollutants provoke damage of the membrane structures and interfere with cellular mechanisms as well as in the gene expression (Koch et al. 1998; Roshchina and Roshchina 2003; Kangasjarvi et al. 2005). At a molecular level, gaseous pollutants have a relatively strong oxidative role that affects biomolecules such as proteins, lipids and nucleic acids that constitute the main material pollen reservoir, and in the end interfere with pollen germination and elongation of the pollen tube (Harrison and Feder 1974).

Ozone is one of the most important atmospheric pollutants, and its increasing concentration in the air is a great concern in Europe (WHO 2000). It is formed by a photochemical reaction with nitrogen dioxide as a precursor (WHO 2000).

Ozone causes short-term acute effects which can include respiratory symptoms and airway inflammation. These effects occur at or close to the upper limits of background concentrations (WHO 2000). These background levels, mainly from anthropogenic origin, are in the range of 0.02–0.035 ppm, but they can reach 0.06–0.07 ppm in 1 h. In Europe, these maximum hourly ozone levels may exceed 0.15 ppm in rural areas and in urban regions 0.18 ppm (WHO 2000).

The European Union fixed safety standards for O₃ that should not be exceeded. According to the Directive 2008/50/EC, the information threshold is 0.085 ppm in 1 h. The alert threshold is 0.114 ppm in 1 h also. The fixed limit value for the protection of human health is 0.061 ppm with a daily maximum of 8-h average threshold, not to be exceeded more than 25 days per calendar year, determined as a 3-year average starting from 2010.

In Europe, the most common pollinosis is caused by pollen from grasses, trees (birch, olive and cypress) and weeds (D'Amato et al. 2007). In Portugal, pollen sensitization studies have demonstrated that in the central region there is a higher sensitization to pollen, particularly to grasses and weeds (Pereira et al. 2006).

In areas with a temperate climate, *P. lanceolata* is an important cause of allergic rhinitis, asthma and conjunctivitis (Gadermaier et al. 2014). Reports

suggest a prevalence in sensitization of up to 36 % among patients with pollen allergy (Gadermaier et al. 2004). *Plantago* is a weed that has now become cosmopolitan, usually grows in neglected gardens and humid meadows and at roadsides. It invades lawns, spreads steadily and is considered an undesirable weed, with no economic or aesthetic value (Gadermaier et al. 2004). Studies have detected 16 different antigens, but only 3 specific allergens of 17, 19 and 40 kDa were isolated from *Plantago* pollen (Asero et al. 2000). Reactivity with this weed is often associated with grass pollen allergy (Gadermaier et al. 2014).

On the other hand, studies on *Salix* are scarce, and no allergens have been identified to date. *Salix* is an ornamental tree widely planted not only in public parks but also in urban green spaces (Cariñanos and Casares-Porcel 2011). Reports in a Swedish multicentre study suggest a prevalence in sensitization of 40 % to *Salix* pollen in patients with birch pollen allergy (de Weger et al. 2013).

The aim of this study was to assess the effect on the viability, protein profile and allergenicity of *Plantago lanceolata* and *Salix atrocinerea* pollen after exposure to two different concentrations of O₃. Airborne pollen concentration was also determined.

2 Materials and methods

2.1 Aerobiological monitoring

Airborne pollen levels were continuously monitored (2003–2013), using a 7-day Hirst-type volumetric spore sampler (Burkard) set on the roof of the Sciences Faculty of the University of Porto (41°11'N, 8°39'W), which is approximately 20 meters above ground level, and calibrated to sample air at 10 L per minute. Pollen was trapped on a Melinex tape coated with silicone oil, which was then cut into daily segments and mounted on slides with a media of glycerol jelly fuchsin. The daily mean concentration of *Plantago* and *Salix* pollen was estimated using an optical microscope (DMLS, Leica) with four full lengthwise traverses at a magnification of ×400. Pollen counts were expressed as the sum of the number of pollen grains per cubic meter of air for a 24-h period. The annual airborne pollen index (API) was used to indicate the total amount of pollen for each year.

2.2 Pollen samples

Pollen from *P. lanceolata* and *S. atrocinerea* were collected during its flowering season in public gardens and sidewalks of the second largest Portuguese city, Porto. The trees and weeds selected were easy to access, free standing and presented flowers with dehiscent anthers. Then, anthers were randomly collected from different parts of the plants. In the laboratory, the anthers collected were dried at 24 °C and gently crushed. The pollen was released by passing it through different grades of sieves to obtain pure pollen. Pollen samples were then stored at −20 °C.

2.3 Patient sera

Data from allergic patients' sera presenting specific IgE levels to pollen extracts, performed by ImmunoCAP FEIA, were acquired from a central hospital in Porto region. Twenty-seven positive ImmunoCAP results to *Plantago lanceolata* pollen were used, and thirty-two were used for *Salix atrocinerea* in immunoblotting experiments. The chosen sera had specific IgE values between low and very high (0.35–54.5 kU_A/L). Each serum represents only one sensitized individual with a regular volume of around 0.5–0.7 mL, which is sufficient for a single experiment. Two sera from non-sensitized patients were used as negative control.

2.4 In vitro exposure to O₃

Pollen samples were artificially exposed to O₃ in an environmental chamber equipped with a solar simulator (Newport Oriel 96000 150 W), a fan (SUNON SF23080AF) to homogenize the air and a temperature and humidity sensors (EBRO EBI20) for monitoring the meteorological condition inside the chamber (Sousa et al. 2012). Pollen samples (200 mg of dry weight) were placed in a tube with both edges closed by a 23-μm pore length mesh (SEFAR PET 1000) and positioned over a fan that impelled the air within the chamber to pass through the tube. Pollen samples were individually exposed to O₃ during 6 h at approximately equal (+) and two times (++) the limit value for the protection of human health in Europe (0.061 ppm; daily maximum 8 h average threshold) based on the European Union Directive

2008/50/EC of May 21, 2008, on ambient air quality and cleaner air for Europe. One blank sample (B) was subjected to the same exposure strategy, but the air inside the chamber was not contaminated with O₃ (Table 1). O₃ was produced using an ozone generator (A2ZS-1GLAB), and its concentration was monitored continuously using an O₃ concentration sensor (AEROQUAL Series 500 sensors) coupled to an OMRON H3DK-S1 timer for controlling the injection of ozone for brief periods in order to offset the high ozone reactivity.

2.5 Pollen fertility

Pollen viability was estimated using the trypan blue stain, a vital dye that interacts with the cell when the membrane is damaged. So, all the non-colored cells are viable. Pollen grains (2 mg) were placed for 2 min in 0.5 mL of 60 % trypan blue and centrifugation for 3 min at 13 200 rpm. Then 40 μL were placed on a microscope slide and observed at light microscope (DMLB, Leica). Pollen viability was determined as the percentage of non-colored pollen relative to the total pollen.

P. lanceolata and *S. atrocinerea* pollen samples were germinated at 27 °C in the dark using a liquid germination medium optimized by the authors (*P. lanceolata* 100 ppm H₃BO₃, 300 ppm CaCl₂ and 16 % sucrose; *S. atrocinerea* 150 ppm H₃BO₃, 400 ppm Proline and 15 % sucrose).

For both viability and germination, five random fields were counted (100 pollen grains per field) using a light microscope (DMLS, Leica).

2.6 Protein extraction and quantification

Pollen grains (50 mg DW) 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 then 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 then quantified colorimetrically with the Coomassie protein assay reagent (Pierce) by the Bradford method. A standard curve estimated using different BSA concentrations (0–2000 μg/mL) was used as a protein standard. Three replicate measurements per extraction were performed.

Table 1 Average and standard deviation of O₃, gas concentration (GC), temperature (T), relative humidity (RH), viability (V), germination (G) and total soluble proteins (TSP) registered during experiments of *P. lanceolata* and *S. atrocinerea* pollen

Pollen	O ₃	GC (ppm)	T (°C)	RH (%)	V (%)	G (%)	TSP (µg/mL)
<i>Plantago lanceolata</i>	B	0.000	26.9 ± 1.3	66.6 ± 1.2	34.8 ± 0.8 ^a	7.6 ± 1.1 ^a	2579 ± 135 ^a
	+	0.065 ± 0.029	27.7 ± 1.5	62.1 ± 1.5	22.6 ± 0.9 ^b	7.2 ± 1.8 ^a	2731 ± 25 ^{a,b}
	++	0.124 ± 0.019	27.5 ± 1.4	68.5 ± 1.9	15.2 ± 0.8 ^c	4.0 ± 1.0 ^b	2852 ± 31 ^b
<i>Salix atrocinerea</i>	B	0.000	28.3 ± 1.3	54.9 ± 1.4	86.0 ± 0.7 ^a	23.2 ± 6.0 ^a	4848 ± 138 ^a
	+	0.061 ± 0.008	26.9 ± 2.0	51.9 ± 1.2	75.4 ± 0.9 ^b	13.2 ± 0.8 ^b	4959 ± 188 ^a
	++	0.118 ± 0.035	27.8 ± 1.2	67.1 ± 1.1	65.8 ± 0.8 ^c	11.2 ± 0.8 ^b	5400 ± 147 ^b

Different letters indicate values statistically significantly different (ANOVA test followed by Tukey HSD post hoc, $p < 0.05$)

2.7 SDS-PAGE and immunoblots

Proteins from pollen extracts were separated in 12.5 % (w/v) polyacrylamide gels under reducing conditions (30 µg/mL per lane). The proteins were then visualized by Coomassie brilliant blue R-250 staining. The molecular weight of protein bands was estimated by comparison with a protein marker (PageRuler™ Plus Prestained Protein Ladder, Fermentas). For immunoblotting analysis, the protein bands separated by SDS-PAGE were electroblotted to nitrocellulose membranes (Protran, Whatman® Schleicher and Schuell, Germany). Transfers were carried out in transfer buffer (192 mM glycine, 25 mM Tris and 20 % methanol) during 2 h at 200 mA. The membranes were saturated during 1 h in a blocking solution (chemiluminescent blocker, Millipore) and then incubated overnight at 4 °C with patient sera diluted 1:10 in blocking solution for the identification of allergens.

After washing, blots were probed with mouse antihuman IgE-HRP (1:2000). The reaction was revealed used Luminata Crescendo Western HRP substrate (Millipore) and imaged by ChemiDoc™ XRS+ System. The antigenic profile bands of the SDS-PAGE and immunoblots were quantified by the software Image Lab 5.2 (Bio-Rad Laboratories). Bands display numerical values that are relative to the reference bands in the blank sample (B). The reference band value is 1.00.

2.8 Statistical analysis

To evaluate the quantitative effects of increasing O₃ levels during the same exposure time on the experimental parameters, a one-way ANOVA was used

followed by the Tukeys' post hoc. The normality of the data was evaluated by Shapiro–Wilk test. Statistical analysis of the experimental results was done using Microsoft Office Excel 2013 spreadsheet and IBM SPSS statistics version 21.

3 Results

3.1 Aerobiological monitoring

Plantago pollen is mainly present in the atmosphere of Porto region from March to September, with most pollen sampled from May to July, while *Salix* pollen is present from February to April, with its peak concentrations being registered in March (Fig. 1a).

Interannual variations in the *Plantago* and *Salix* pollen index were observed (Fig. 1b). The *Plantago* annual pollen index was maximal in 2009 (402.8) and minimal in 2004 (97.7) with an average of 205.8. The *Salix* annual pollen index was maximal in 2008 (76.4) and minimal in 2009 (6.4) with an average of 31.2.

3.2 Pollen fertility

Table 1 shows viability and germination rates of *Plantago* and *Salix* pollen after O₃ exposure. For both pollen types, viability significantly decreased with increasing concentrations of O₃.

Results of the germination tests show that the blank pollen samples presented always higher germination rates than the samples exposed to the pollutant. However, in *P. lanceolata* pollen the germination decrease was only statistically significant when pollen was exposed to O₃ concentrations at two times above

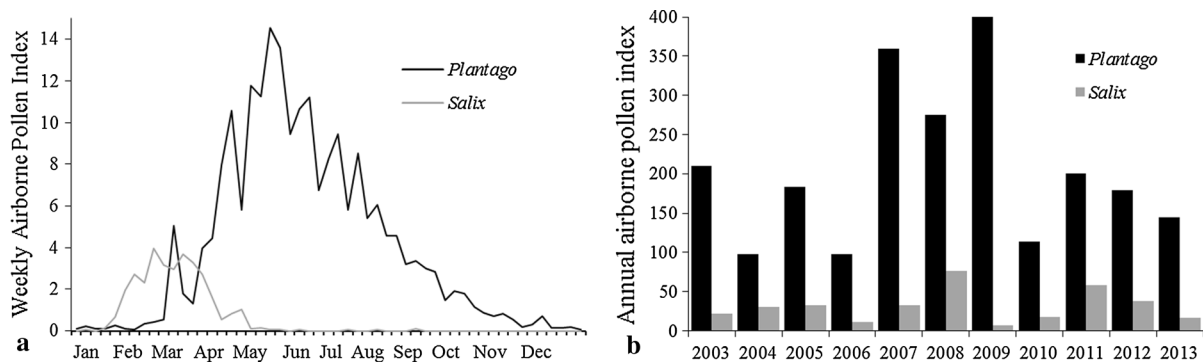


Fig. 1 Average weekly airborne pollen concentration (a) and annual airborne pollen index (b) of *Plantago* and *Salix* in the Porto region (2003–2013)

the limit for human health protection. In *S. atrocinerea* pollen, germination significantly decreased regardless of the gas concentrations tested.

3.3 Protein extraction and quantification

The total soluble protein (TSP) content of the exposed O_3 pollen samples increased when compared with the blank samples (Table 1). However, this increase was only statistically significant when samples of both pollen types were exposed to two times the 8-h limit concentration for human health protection.

3.4 SDS-PAGE and immunoblots

The analysis of the SDS-PAGE of *Plantago* and *Salix* samples shows a polypeptide profile ranging from 10 to 70 kDa (Fig. 2). No visible differences were observed between the polypeptide profile of the blank and exposed samples.

In *Plantago lanceolata*, out of 27 sera, 71 % reacted to protein bands of 30–31 kDa; 54 % reacted to protein bands of 34–35 kDa; 50 % reacted to protein bands of 53–54 kDa; 32 % reacted to protein bands of 40–42 kDa and 18 % reacted to protein bands of 16–17, 15 kDa (Fig. 3).

In *Salix atrocinerea*, out of 32 sera, 72 % reacted to protein bands of 54 kDa; 63 % reacted to protein bands of 31 kDa; 53 % reacted to protein bands of 49–50 kDa; 28 % reacted to protein bands of 24–25 kDa; 25 % reacted to protein bands of 72–74 kDa and 9 % reacted to protein bands of 10–11 kDa (Fig. 4).

Pollen allergenic changes were assessed according to the binding affinity to specific IgE. Increased optical densities were observed in protein bands of the pollen exposed when compared with the blank pollen samples.

4 Discussion

In the Porto region, daily airborne *Plantago* pollen was detected during late spring and summer at a daily average concentration of around 14 grains/m³. This value is close to the airborne concentrations registered in Northwestern Spain (González-Parrado et al. 2015; Iglesias-Otero et al. 2015). However in *Salix* pollen, it was detected during late winter and early spring at average concentration lower than 4 grains/m³. Both values are considered low risk for the allergenic reactions (Portuguese Aerobiology Network); however, that does not mean that the allergenic reactions will be less. The special allergenic ability of *Plantago* pollen grains (Minero et al. 1998) has been demonstrated, and even at low concentrations, its proteins cause significant allergic symptoms (D'Amato et al. 1991). In fact, when people monosensitized to *Plantago* pollen begin to show respiratory symptoms, few pollen grains are present in the atmosphere (González-Parrado et al. 2014). In Germany, this pollen type is recognized as one of the major allergens (D'Amato et al. 1991). In Barcelona, *Plantago* is ranked third in herbaceous plants, in terms of pollinosis, behind *Parietaria* and *Artemisia* (Santiago et al. 2002). Also, due to cross-reactivity phenomena (Gadermaier et al.

Fig. 2 SDS-PAGE of total soluble proteins of *Plantago lanceolata* and *Salix atrocinerea* pollen exposed to O₃ (a). Immunoblots of IgE-reactive bands of *Plantago lanceolata* (b) and *Salix atrocinerea* (c), these two were taken as representative of the overall immunoblots performed. Labels above the lanes represent gas concentration level: B—0 ppm, +—0.061 ppm, ++—0.122 ppm

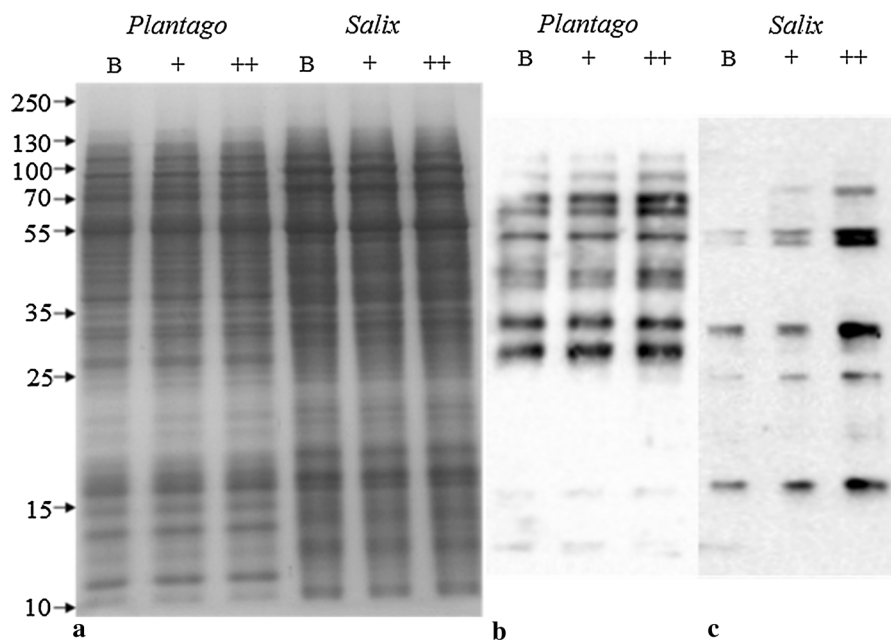
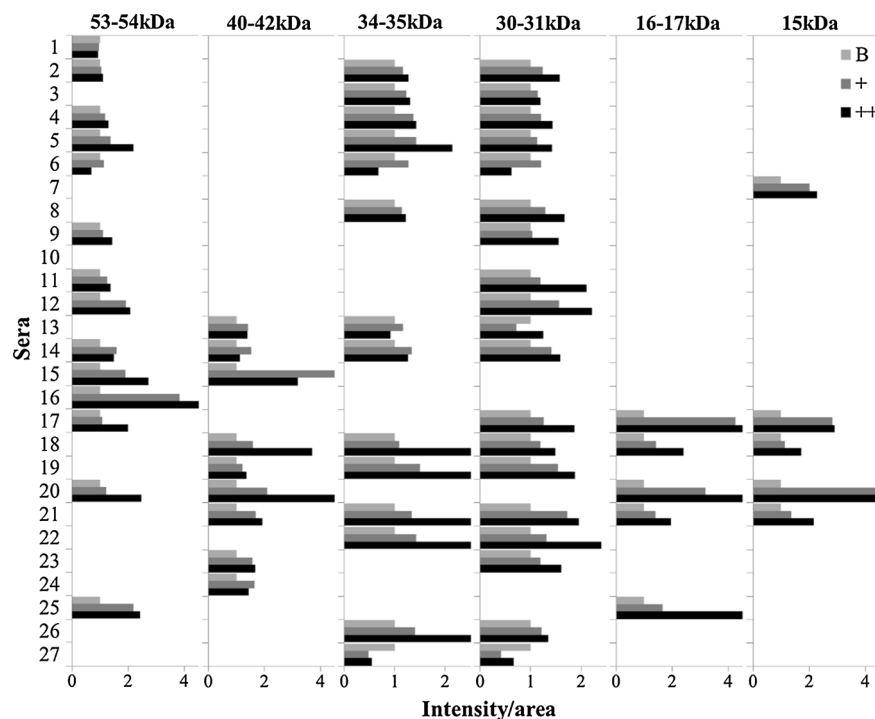


Fig. 3 Optic densities (O.D.) of the most prominent IgE-reactive bands of *Plantago lanceolata* pollen with a panel of 27 sera from different sensitized individuals to pollen. O₃ concentration level: B—0 ppm, +—0.061 ppm, ++—0.122 ppm



2004; Castro et al. 2007; Radauer and Breiteneder 2006), the concomitant presence of high airborne pollen levels from other allergenic sources, such as grasses, olives and *Parietaria*, result in an increase in

the exposure period as well as in the allergenic pollen load.

Regional levels of airborne *Salix* pollen are often low (Jones and Harrison 2004) and, in Europe, no

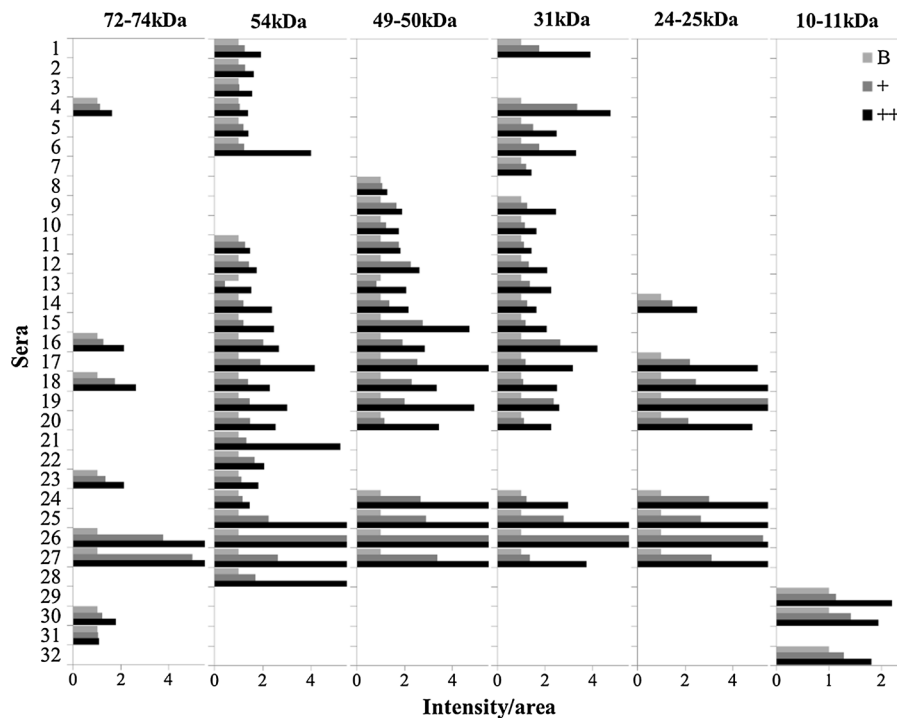


Fig. 4 Optic densities (O.D.) of the most prominent IgE-reactive bands of *Salix atrocinerea* pollen with a panel of 32 sera from different sensitized individuals to pollen. O₃ concentration level: B—0 ppm, +—0.061 ppm, ++—0.122 ppm

threshold values are published (de Weger et al. 2013). Cross-reactivity with birch pollen-sensitive people has been reported (de Weger et al. 2013). Cross-reactivity with *Fraxinus*, *Platanus* or *Populus* pollen has also been reported (Kadocska et al. 1993).

Pollutant gases, such as O₃, have been pointed out to affect several pollen features. Several studies have reported a decrease in pollen viability and germination in several plant species after pollen exposure to either in vitro or in vivo conditions to different concentrations of a specific pollutant gas (Sousa et al. 2012; Cuinica et al. 2014; Wolters and Martens 1987).

The exposure of the *P. lanceolata* and *S. atrocinerea* pollen to ozone concentrations, corresponding to the limit values for human health protection and two times that value, affected significantly the fertility of the pollen. Indeed, the analysis of the viability of the pollen after exposure shows significant decrease, which is more accentuated in *Plantago*. Also the pollen germination after exposure to O₃ significantly decreased, being in *Plantago* about 47 % and in *Salix* about 52 %. These results agree with those obtained in various in vitro studies exposing pollen to pollutant

gases (Chichiricco and Picozzi 2007; Cuinica et al. 2013; Cuinica et al. 2014). In fact, ozone can have deleterious effects on cell membranes at a subcellular level (Omasa 2002) and may even cause organelle disconnection from the plasma membrane. Previous studies showed that air pollutants are a stress factor for plant species affecting physiological processes in pollen (Helander et al. 1997). At the molecular level, O₃ has strong oxidative activity that affects biomolecules of the cell compartments (Omasa 2002), such as proteins, lipids and nucleic acids. Such oxidative potential may interfere with pollen tube development (Harrison and Feder 1974), so it is likely that oxidative damage could be responsible for the reduction in pollen fertility observed in our study.

In addition to affecting the fertilization, exposure to O₃ also significantly affects the total soluble proteins of pollen when exposed to two times the O₃ limit concentration for human health protection. The same conclusion was obtained for *Ostrya carpinifolia* and *Carpinus betulus* pollen when exposed to O₃ during 2 days (Cuinica et al. 2014). However, many authors have reported a decrease in total soluble proteins of

pollen after exposure not only to O₃ but also to other pollutant gases (Majd et al. 2004). Taken together, these studies suggest that protein content after pollutant exposure may be dependent on plant species and gas concentrations.

Even though no electrophoretic profile changes were observed in our experiment, an increase in IgE reactivity of patient sera to pollen extracts was observed with the increasing concentration of O₃, which in turn can be related with increasing allergenicity. We identified six different prominent bands in both plants. Two of these bands were common to both species. Some of the bands identified in *Plantago* can correspond to allergens already identified in previous works (Asero et al. 2000; Sousa et al. 2014). So far, only three *Plantago* pollen allergens were identified: Pla 1 1 (17 kDa unglycosylated protein), reported as a major allergen, Pla 1 cytochrome C, a cytochrome c protein (Matthews et al. 1988), Pla 1 CBP, a calcium binding protein (Grote et al. 2008). Furthermore, Asero et al. (2000) reported a marked IgE reactivity at about 40 kDa on an immunoblot of a patient serum monosensitized to *Plantago* pollen, which can indicate a novel allergen. In *Salix*, almost all the bands identified correspond to pollen immunological profile reported in Ribeiro et al. (2009). No identification and characterization of its allergens have been performed, and the common bands with other tree pollen immunological profiles (Ribeiro et al. 2009, 2013) and to *P. lanceolata* could indicate the existence of cross-reactivity among pollen. Allergic cross-reactivity to tree pollen from various plants has been reported (Mothes and Valenta 2004). In fact, cross-reactivity can be linked with the ability of IgE antibodies, originally raised against a given allergen, to bind to homologous molecules originating from a different allergen source (Hauser et al. 2010).

In our study, the increase in the optical densities observed is more marked in *Salix* than in *Plantago* pollen, which could suggest that O₃ may have a lower impact on pollen allergenicity in *Plantago*. This less influence of O₃ in *Plantago* was also reported for ragweed pollen, showing no change in IgE reactivity when exposed to O₃ (Pasqualini et al. 2011). Also, Rogerieux et al. (2007) found a decrease in reactivity in *Phleum pratense* pollen, after exposure to ozone, which could be a result of allergen loss and/or PTMs affecting allergen recognition by IgE. Post-translational

modification by ozone can enhance the allergenic potential of proteins. This process of post-translational modification can be induced by ozone, which reacts with organic compounds that contain reactive oxygen atoms or groups, in our case with pollen proteins, resulting in the formation of reactive oxygen intermediates (ROIs) (Shiraiwa et al. 2012b), which play a key role in the formation of toxic components. This post-translational modification by ozone can change the protein function which can lead to an increase or decrease in the allergenic potential of proteins (Shiraiwa et al. 2012a).

In *Salix* pollen, the increase in IgE reactivity observed in our study is in accordance with the results obtained for other tree pollens, for example in a study of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* (Cuinica et al. 2015), and *Acer negundo* and *Quercus robur* (Ribeiro et al. 2014). However, for *Platanus x acerifolia*, an IgE reactivity decreased with the exposure to O₃ was reported (Ribeiro et al. 2014).

This study shows that *P. lanceolata* and *S. atrocinerea* pollens contain several proteins that can be highly allergenic which can be modified by ozone present in the atmosphere and can lead to an increase in the allergenicity. The fertilization analysis presented here revealed that even at the limit concentration for human health, the viability and germination rate of both *Plantago* and *Salix* pollen were affected by the ozone.

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