

Ozone effects on soluble protein content of *Acer negundo*, *Quercus robur* and *Platanus* spp. pollen

H. Ribeiro · L. Duque · R. Sousa · I. Abreu

Received: 26 September 2012 / Accepted: 18 January 2013
© Springer Science+Business Media Dordrecht 2013

Abstract In this study, the effect of three different ozone (O₃) levels on soluble protein content of *Acer negundo*, *Quercus robur* and *Platanus* spp. pollen was investigated. Pollen was directly collected from each plant and exposed to O₃ values below, equal and four times the limit for human health protection in an environmental chamber system. With this chamber, it was possible to control and reproduce the O₃ levels between different experiments. Total protein content was determined colorimetrically with Coomassie protein assay reagent (Pierce) by the Bradford method. Results showed that O₃ has significant effects on pollen causing a decrease in soluble proteins that seem to be dependent on species and gas concentration. Also, the existence of reproducibility in the effects of O₃, within the same concentration, on the protein content of pollen was verified.

Keywords In vitro exposure · Environmental chamber · Pollen · Protein

H. Ribeiro (✉) · L. Duque · R. Sousa · I. Abreu
Grupo do Ambiente do Centro de Geologia,
Universidade do Porto, Porto, Portugal
e-mail: helenaribeiro@fc.up.pt

I. Abreu
e-mail: ianoronh@fc.up.pt

I. Abreu
Departamento de Biologia, Faculdade de Ciências,
Universidade do Porto, Rua do Campo Alegre, s/n,
4169-007 Porto, Portugal

1 Introduction

Environmental exposure is pointed to be a critical factor for the increasing prevalence of respiratory allergic diseases and includes many diverse causes such as air quality (Ring 2012). The increase in pollen-related respiratory allergies has been associated with the interaction between pollen allergens and certain levels of gaseous components of the aerosol such as O₃, SO₂, NO₂ and CO (D'Amato 2011).

In Europe, tropospheric ozone is one of the most important air pollutants causing severe health effects and vegetation damage, both as an active agent and through ozonolysis-generated compounds. It is formed at ground level by photochemical reactions whose precursors are the gases nitrogen oxides (NO_x), carbon monoxide (CO) and volatile organic compounds (VOC). In the period 1997–2009, it was estimated that between 13 and 61 % of the EU and EEA-32 urban population were exposed to ozone concentrations higher than the EU limit value for human health protection (EEA 2011). Current safety standards for O₃ specify for health protection a daily maximum 8-h average threshold of 120 µg/m³ (0.061 ppm), determined as a 3-year average starting from 2010 (EEA 2011; EU 2008).

Pollen grains contain many different types of proteins with specific functions such as recognition reactions with stigma. Also, some pollen proteins are well-characterized antigens responsible for immediate hypersensitivity reactions (Rawat et al. 2000). While

airborne, pollen exposed to pollutants such as ozone can possibly suffer modifications in its protein content and structure. However, there are still some questions regarding the influence of gas concentration levels and the plant species exposed on pollen's soluble protein content.

The aim of this investigation was to study the influence of different O₃ levels on pollen's soluble protein content of three trees: *Acer negundo*, *Quercus robur* and *Platanus* spp. Also, the reproducibility of exposure conditions and pollen's soluble protein content were evaluated.

2 Materials and methods

2.1 Pollen samples

Pollen from *A. negundo*, *Q. robur* and *Platanus* spp. was collected in Porto region in public gardens or sidewalks, during the flowering period. The trees selected were easy to access, preferably young and freestanding, with abundant branches, and presented flowers with dehiscent anthers. In each tree species anthers were randomly collected from all quadrants of the tree in different branches. After removal of other plant parts, the anthers were dried at 24 °C and 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.

2.2 O₃ pollen exposure

An environmental chamber system was developed by our research group (Sousa et al. 2012) to expose pollen samples to several atmospheric pollutant gases. This chamber is equipped with a solar simulator (Newport Oriel 96000 150 W), a fan (SUNON SF23080AF) to homogenize the air inside the chamber and temperature and relative humidity sensors (EBRO EBI20 sensor).

Each pollen sample (200 mg of dry weight—DW) was placed in a tube with both edges closed with a 23-μm-pore length mesh (SEFAR PET 1000). This tube was then placed over a 12 V DC fan that impelled the air within the chamber to pass through the tube. Pollen was exposed to ozone (O₃) for 6 h at three different concentrations, chosen based on the European Union Directive 2008/50/EC of May 21,

2008 (EU 2008), on ambient air quality and cleaner air for Europe. Pollen samples were individually exposed to approximately half (+), equal (++) and four times the standard limit (+++) value. The half-value is similar to the O₃ average observed in the city of Porto (Sousa et al. 2008), and the four times the standard limit values are close to the O₃ maximum values registered in northeast Portugal (Carvalho et al. 2010). O₃ concentrations were attained using an ozone generator (A2ZS-1GLAB ozone generator), and its concentrations were continuously monitored at every minute using an O₃ gas concentration sensor (AERO-QUAL Series 500 sensors). Two replicate exposures were performed for each O₃ level/pollen type (A and B) pair. Control pollen non-exposed to the chamber conditions was used.

2.3 Protein extraction and estimation

Protein extracts were prepared by incubating pollen in 1:20 (w/v) phosphate-buffered saline at pH 7.4 with continuous stirring for 4 h at 4 °C. The suspension was then centrifuged at 16,100g for 30 min at 4 °C. The supernatant was filtered through a 0.45-μm Millipore filter and centrifuged once again. For each sample, which included the control one, two replicate extractions were performed.

The soluble protein content of all pollen extracts was estimated colorimetrically with Coomassie protein assay reagent (Pierce) by Bradford's method (Bradford 1976). 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.

2.4 Statistical analysis

Protein data presented normal distribution (tested using a Shapiro–Wilk test, $n = 6$), while O₃ did not (tested using a Kolmogorov–Smirnov test, $n = 360$) (data not shown). So, O₃ data were transformed using $\sqrt{y_i} + \sqrt{y_i + 1}$, where y_i corresponds to each O₃ value registered, since values were small and some are null.

Student's t test was used to compare the O₃ levels and soluble protein values between replicate experiments within the same exposure level, to evaluate the reproducibility of the assays. The same test was used

Table 1 O₃ fumigation levels and *t* test *p* value associated with the differences between the doubling assays of the same O₃ concentration level (ppm)

	Level	Min	Max	Avg.	SD	<i>p</i> value*
<i>A. negundo</i>	A +	0.00	0.10	0.029	0.02	0.68
	B +	0.00	0.11	0.030	0.03	
	A ++	0.00	0.21	0.057	0.04	0.29
	B ++	0.00	0.13	0.058	0.03	
	A +++	0.09	0.40	0.227	0.07	0.68
	B +++	0.09	0.46	0.233	0.08	
<i>Q. robur</i>	A +	0.00	0.06	0.029	0.01	0.07
	B +	0.00	0.07	0.031	0.01	
	A ++	0.01	0.18	0.057	0.03	0.65
	B ++	0.01	0.13	0.058	0.03	
	A +++	0.11	0.48	0.233	0.07	0.73
	B +++	0.11	0.46	0.235	0.06	
<i>Platanus</i> spp.	A +	0.00	0.09	0.029	0.02	0.14
	B +	0.00	0.06	0.030	0.01	
	A ++	0.02	0.10	0.060	0.02	0.73
	B ++	0.00	0.11	0.060	0.02	
	A +++	0.13	0.38	0.227	0.05	0.08
	B +++	0.09	0.45	0.235	0.07	

A and B are replicates of the same condition

+ Half the limit value; ++ limit value; +++ four times the limit value

* Values lower than 0.05 indicate the rejection of the null hypothesis at the 95 % confidence level

for assessing the significance of quantitative changes in protein content, for each pollen species, for the different levels of O₃ tested. Values of $p < 0.05$ were considered significant.

3 Results

3.1 Reproducibility of the exposure assays

The O₃ levels tested and the *p* values associated with the *t* test performed for the equality of O₃ level means within each concentration assayed are displayed in Table 1. Reproducibility between exposure conditions within each of the three concentrations tested was observed since no significant differences were observed (*p* values always >0.05).

Considering the results of the *t* test performed for the equality of the average soluble protein content between the two exposure replicates of the same O₃

levels (assay A vs. assay B), no significant differences were observed (*p* value between 0.218 and 0.809).

3.2 Influence of O₃ on pollen's total soluble protein content

In Table 2 is shown the average total soluble protein content of *A. negundo*, *Q. robur* and *Platanus* spp. after exposure to the three levels of O₃. In *A. negundo* pollen, no significant differences were observed in protein content when pollen was exposed to half the limit and limit concentration level for human health protection. Only values four times the limit concentration seem to cause a significant decrease in protein content.

For *Q. robur* and *Platanus* spp., pollen exposure to O₃, regardless the concentration, reduced significantly the protein content of these species. For *Platanus* spp. pollen, below, limit and four times the limit values for human health protection produced similar reduction in protein content. However, in *Q. robur* pollen, a significant reduction in protein content was also observed between the three exposure levels tested, showing that increasing O₃ concentrations lead to decrease in protein content.

4 Discussion

In this work statistical test was performed to compare the differences between pollen's soluble protein content after exposure to ozone in order to test whether there were any statistically significant differences compared with the control samples. Also, two replicates were performed, and statistical test was applied to compare the differences between exposure levels and pollen's soluble protein content between replicates.

The statistical analysis performed to ascertain the ability to reproduce the exposure experiments showed that with the environmental chamber, it was possible to control the O₃ levels in order to obtain concentrations below, equal and four times the limit values for human health protection in Europe as well as to reproduce these levels. Also, the average protein content determined in the two exposure repetitions for each O₃ concentration level was statistically significantly similar. This indicates that it is possible to reproduce effectively the effects of O₃, within the

Table 2 *t* test *p* values for differences in average pollen protein content between each exposition level

Species	Protein content (µg/ml)	<i>t</i> test <i>p</i> value*			
			+	++	+++
<i>A. negundo</i>	5,178.6	C	0.738	0.755	0.003
	5,101.7	+	1.000	0.967	0.000
	5,092.5	++		1.000	0.002
	4,364.0	+++			1.000
<i>Q. robur</i>	4,409.1	C	0.005	0.000	0.000
	4,222.3	+	1.000	0.058	0.001
	4,113.2	++		1.000	0.051
	3,978.2	+++			1.000
<i>Platanus</i> spp.	3,457.3	C	0.000	0.002	0.004
	2,851.0	+	1.000	0.172	0.807
	2,964.3	++		1.000	0.521
	2,880.2	+++			1.000

C control; + half the limit value; ++ limit value; +++ four times the limit value

* Values lower than 0.05 indicate the rejection of the null hypothesis at the 95 % confidence level

same gas concentration, on the protein content of exposed pollen samples.

Some broader studies have compared the protein content of the pollen of *Lagerstroemia indica* (Rezanejad 2009), *Canna indica* (Majd et al. 2004) and *Chenopodium album* (Sousa et al. 2011) from polluted areas with less polluted ones. A significant decrease in soluble protein concentrations per gram of pollen in polluted areas was found compared to the control samples. Moreover, even when control pollen was exposed for 10 and 20 days in the polluted areas, a significant decrease in its total protein content was observed (Rezanejad 2009; Majd et al. 2004). Sousa et al. (2012) showed that in vitro exposure of *A. negundo* pollen to SO₂ and NO₂ levels, around the standard limit values for the human health protection, can led to modifications in the soluble protein content. These studies support the idea of an interaction between airborne pollen and air pollutants outside the human organism. In fact, our investigation indicates that the protein content in the pollen is affected after its in vitro exposure to O₃ and prior to its inhalation by the human organism.

In our study, it was also shown that O₃ affects protein content of *A. negundo* pollen but a significant decrease was only observed for O₃-exposed pollen at four times above the limit values. In a study exposing

ragweed pollen to O₃ at a concentration above the standard limit values for the human health protection (0.100 ppm) for 5 h per day during 7 consecutive days, no significant changes in protein release from exposed versus non-exposed pollen were described (Pasqualini et al. 2011). However, the hypothesis that equivalent levels to those used in our study would influence protein release cannot be ruled out as O₃ levels used in the aforementioned study were lower than the ones tested in the present study.

On the contrary, for *Q. robur* and *Platanus* spp. pollen, the exposure to ambient levels of O₃ was sufficient to induce a significant decrease in protein content. Moreover, some differences in O₃ influence between these last two species were also observed. In *Q. robur* pollen, a significant decrease in protein content values with escalating concentration levels was observed, while in *Platanus* spp. pollen, no significance in average protein content was observed regardless of the O₃ concentrations tested. These results show that changes in soluble protein content in pollen after O₃ exposure may be dependent on species and gas concentration.

In this study was reported a decrease in pollen's soluble protein content after in vitro exposure to ozone. Sousa et al. (2012) showed that a decrease in pollen's total soluble protein content was not related with less pollen allergenicity. Furthermore, Pasqualini et al. (2011) showed for *Ambrosia artemisiifolia* that ozone exposure reduced pollen viability and induced a significant enhancement of the ROS-generating enzyme NAD(P)H oxidase. It would be interesting in the future to ascertain the consequences of the pollen's soluble protein content modifications after ozone exposure on the tested species related to pollen allergenicity, biochemistry and germination.

Acknowledgments This work was supported by FEDER funds through the operational program competitiveness factors (COMPETE) and National funds through FCT—Foundation for Science and Technology in the project “Effects of atmospheric non-biological pollutants on pollen grains” (Ref^a PTDC/AAC-AMB/102796/2008) and PEst-OE/CTE/UI0039/2011- UI 39. Ribeiro, H. benefits from a scholarship (SFRH/BDP/43604/2008) financed by QREN-POPH and FCT.

References

- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing

- the principle of protein dye binding. *Analytical Biochemistry*, 72(1–2), 248–254.
- Carvalho, A., Monteiro, A., Ribeiro, I., Tchepel, O., Miranda, A. I., Borrego, C., et al. (2010). High ozone levels in the northeast of Portugal: Analysis and characterization. *Atmospheric Environment*, 44(8), 1020–1031.
- D'Amato, G. (2011). Effects of climatic changes and urban air pollution on the rising trends of respiratory allergy and asthma [Review]. *Multidisciplinary Respiratory Medicine*, 6(1), 28–37.
- EEA. (2011). *Air quality in Europe—2011 report*. European environmental agency technical report no 12/2011, p. 80.
- EU. (2008). Directive 2008/50/EC of the European parliament and of the council of 21 May 2008 on ambient air quality and cleaner air for Europe, OJ L 152, 11.6.2008 (<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:152:0001:0044:EN:PDF>). Accessed September 21, 2012.
- Majd, A., Chehregani, A., Moin, M., Gholami, M., Kohno, S., Nabe, T., et al. (2004). The effects of air pollution on structures, proteins and allergenicity of pollen grains. *Aerobiologia*, 20(2), 111–118.
- Pasqualini, S., Tedeschini, E., Frenguelli, G., Wopfner, N., Ferreira, F., D'Amato, G., et al. (2011). Ozone affects pollen viability and NAD(P)H oxidase release from *Ambrosia artemisiifolia* pollen. *Environmental Pollution*, 159(10), 2823–2830.
- Rawat, A., Singh, A., Singh, A. B., Gaur, S. N., Kumar, L., Roy, I., et al. (2000). Clinical and immunologic evaluation of *Cedrus deodara* pollen: A new allergen from India. *Allergy: European Journal of Allergy and Clinical Immunology*, 55(7), 620–626.
- Rezanejad, F. (2009). Air pollution effects on structure, proteins and flavonoids in pollen grains of *Thuja orientalis* L. (Cupressaceae). *Grana*, 48(3), 205–213.
- Ring, J. (2012). Davos declaration: Allergy as a global problem. *Allergy: European Journal of Allergy and Clinical Immunology*, 67(2), 141–143.
- Sousa, R., Cruz, A., Ribeiro, H., & Abreu, I. (2011). Impact of urbanization level on *Chenopodium album* pollen: Morphological and immunochemical data. *Revista Portuguesa de Imunoalergologia*, 19(1), 33–41.
- Sousa, R., Duque, L., Duarte, A. J., Gomes, C. R., Ribeiro, H., Cruz, A., et al. (2012). In vitro exposure of *Acer negundo* pollen to atmospheric levels of SO₂ and NO₂: Effects on allergenicity and germination. *Environmental and Science Technology*, 46, 2406–2412.
- Sousa, S. I. V., Martins, F. G., Pereira, M. C., Alvim-Ferraz, M. C. M., Ribeiro, H., Oliveira, M., et al. (2008). Influence of atmospheric ozone, PM₁₀ and meteorological factors on the concentration of airborne pollen and fungal spores. *Atmospheric Environment*, 42(32), 7452–7464.