Aerobiologia
https://doi.org/10.1007/s10453-020-09676-2

ORIGINAL PAPER

Air pollutants NO_2- and O_3-induced *Dactylis glomerata* L. pollen oxidative defences and enhanced its allergenic potential

A. Galveias · R. Arriegas · S. Mendes · H. Ribeiro · I. Abreu · A. R. Costa · C. M. Antunes

Received: 2 April 2020 / Accepted: 3 November 2020
© Springer Nature B.V. 2020

Abstract Air pollutants impact airborne pollen biochemistry. Oxidative damage to lipids, proteins and nucleic acids or protein nitration are among ozone (O_3) and nitrogen dioxide (NO_2) described deleterious effects possibly causing pollen physiology damage and enhanced allergenic activity, contributing to aggravate pollen driven respiratory allergy in urban areas. The goal of this research was to evaluate the effects of O_3, NO_2, alone and combined, on *Dactylis glomerata* pollen reactive oxygen species scavenging enzymes, on pollen germination and their potential contribution to the allergenicity. *D. glomerata* pollen was in vitro exposed to pollutants. Protein extracts were prepared and superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activities were evaluated. Western blot with pooled sera or with IgG against group 5 allergens and profilin was performed. Pollen germination capacity was increased by NO_2 and was unaffected by O_3 or $O_3 + NO_2$ but showed longer pollen tubes in the latter. Exposure to O_3 did not affect SOD activity but induced a twofold increase in catalase activity. SOD activity was twofold higher in pollen exposed to NO_2. Exposure to $O_3 + NO_2$ induced a twofold and fivefold increase of SOD and catalase activities, respectively. Pollen GPx was unaffected by the pollutants. IgE-recognition of proteins in the molecular weight range of 42–57 kDa were amplified by NO_2 and $O_3 + NO_2$ and O_3 amplified proteins with molecular weight of 13 (profilin), 29 (Group 5), and 31 kDa. Taken together, these results show that pollen oxidative defences are activated by common air pollutants affecting both its germination capacity and its allergenic activity.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10453-020-09676-2) contains supplementary material, which is available to authorized users.

A. Galveias · R. Arriegas · A. R. Costa · C. M. Antunes (✉)
Institute of Earth Sciences (ICT) and Department of Chemistry, School of Sciences and Technology, University of Évora, Rua Romão Ramalho, 59, 7000-671 Évora, Portugal
e-mail: cmma@uevora.pt

S. Mendes
Department of Chemistry, School of Sciences and Technology, University of Évora, Rua Romão Ramalho, 59, 7000-671 Évora, Portugal

H. Ribeiro
Institute of Earth Sciences (ICT) and Department of Geosciences, Environment and Spatial Planning, Faculty of Sciences, University of Porto, Rua Do Campo Alegre, 687, 4169-007 Porto, Portugal

I. Abreu
Institute of Earth Sciences (ICT) and Department of Biology of the Faculty of Sciences, University of Porto, Rua Do Campo Alegre, s/n, 4169-007 Porto, Portugal

Published online: 27 November 2020

Keywords Pollen germination · Superoxide dismutase · Catalase · Atmospheric pollutants · Aeroallergens · Allergenicity

Abbreviations

ROS Reactive oxygen species
SOD Superoxide dismutase
GPx Glutathione peroxidase
tBH *t*-Butyl hydroperoxide

1 Introduction

Motor vehicles emissions, from burning hydrocarbons, produce nitrogen dioxide (NO_2). With available UV light, photodissociation of NO_2 can produce nitrogen monoxide (NO) and atomic oxygen (O), which is very reactive and attaches quickly to oxygen (O_2) to form ozone (O_3). This way, tropospheric O_3 can be formed indirectly from pollution of NOx. The short half-life of O_3 causes its break down to $O_2 + O$, which in turn, can bind to natural nitrogen (N_2) or to NO to again form NO_2. Unlike naturally formed O_3, when O_3 is formed from smog, an "ozone cycle" begins that is difficult to break, where NO and NO_2 participate as catalysts, causing ozone to increase in our environment to unhealthy levels (Zumdahl 2009). Portugal has very intense solar radiation levels, so in the presence of the ozone precursors, which can be transport from the urban areas of Porto and Lisbon to the south and interior part of Portugal by the N–NW dominant winds, ozone levels can reach the threshold limit imposed by the EC directives, presenting risk for vegetation protection (Monteiro et al. 2007).

Airborne pollen during its transport comes in contact with a variety of atmospheric chemicals, including the common air pollutants O_3 and NO_2, which impact airborne pollen at several levels: affecting germination capacity (Gottardini et al. 2004) and viability (Cuinica et al. 2014; Pasqualini et al. 2011), morphology and cell wall structure (Chehregani et al. 2004) and protein content or release (Ribeiro et al. 2014), as well as inducing chemical modification of specific biomolecules. On one side, NO_2, alone or in combination with O_3, may contribute to protein nitration (Ghiani et al. 2016; Gruijthuijsen et al. 2006), affecting protein/enzymatic function. On the other side, the strong oxidizing potential of O_3 may affect pollen redox balance (Pasqualini et al. 2011), and oxidative damage to lipids, proteins and nucleic acids are among O_3 described deleterious effects (Iriti and Faoro 2007). Moreover, in nature it is expected that the airborne pollen will be exposed to both pollutants simultaneously and other synergistic effects may be expected, since the presence of O_3 increases NO_2 absorption (Chassard et al. 2015) favouring the nitration of proteins (Franze et al. 2005). Nevertheless, the effect of these pollutants on pollen, alone or in combination, is not fully elucidated and are, probably, pollen type specific.

The pollen of grasses is very abundant in the spring period in Portugal, corresponding to an increasing solar exposure (UV radiation), which induces, with the adequate catalysts present, elevated O_3 levels in the atmosphere (Bortoli et al. 2009). Additionally, the high humidity levels characteristic of the season, above 60% even in inland drier areas (Miranda et al. 2001), facilitate the uptake of pollutants by the pollen (Chassard et al. 2015). So, it is relevant to know the effects of atmospheric pollutants on this pollen type, particularly due to its high allergic potential.

Reactive oxygen species (ROS) are important for regulating many aspects of the life cycle and environmental response mechanisms of plants (growth, development, response to biotic and abiotic environmental stimuli). However, stress can lead to excessive ROS production, causing progressive oxidative damage. Whether ROS would serve as signalling molecules or could cause oxidative damage to the tissues depends on the delicate equilibrium between ROS production, and their scavenging (Bailey-Serres and Mittler 2006). In pollen, as in other plant tissues, ROS, directly or indirectly derived from the exposure to pollutants, are scavenged by enzymatic and non-enzymatic antioxidant defensive mechanisms. In this work, we investigate the action of superoxide dismutase (SOD), which constitutes the first line of defence against ROS, and catalyses the dismutation of the superoxide radical ($O_2^{-\cdot}$) into either O_2 or H_2O_2, which can also be damaging (causing lipids or proteins peroxidation) and is degraded by other enzymes such as catalase or glutathione peroxidase (GPx), which also will be investigated.

Environmental pollutants can affect allergic disease in several ways, for instance as adjuvant factors in sensitization or as triggering factors in allergic disease onset. Environmental pollutants can also affect

allergen exposure features, influencing either sensitization or the onset and/or magnitude of allergic disease (Ring et al. 2001).

The goal of this research was to evaluate the effects of O_3, NO_2, alone and combined, on physiological aspects and on allergenicity of *D. glomerata* pollen. In order to perform this evaluation, we have investigated the effect of the above gaseous pollutants on: (i) pollen germination ability; (ii) the major defences against oxidative stress, the ROS scavenging enzymes SOD, catalase and GPx; (iii) the allergen expression patterns identified by IgE immunorecognition. *D. glomerata* pollen was exposed to the air pollutants in controlled conditions, using an environmental chamber.

2 Material and methods

2.1 Pollen collection

D. glomerata pollen anthers were collected during the flowering season (spring; June 2018) in a rural area of North of Portugal and were dried at 27 °C. Pollen was released by gently crushing the anthers and passed through 0.063 mm sieves to isolate the pollen samples that were stored at − 20 °C after exposure to the different treatments (Sect. 2.2). Figure 1 depicts the experimental design.

2.2 In vitro pollen exposure to O_3 and NO_2

Pollen samples were exposed to O_3, NO_2 or $O_3 + NO_2$ 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) (chamber and method details may be consulted in 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-µm pore length mesh (SEFAR PET 1000). This tube was then placed over a fan that impelled the air within the chamber to pass through. Pollen was exposed to pollutants during 6 h, at an average concentration equal to the current atmospheric limit value for the human health protection according to the European Union Directive 2008/50/EC of 21 May 2008 on ambient air quality and cleaner air for Europe (O_3 daily 8-h mean—120 µg/m^3; NO_2 1 h limit—200 µg/m^3). A pollen sample exposed under the same conditions to unpolluted air was used as control. The 6 h exposure time was used as a model for the period corresponding to the highest solar irradiation (around 12 o'clock).

2.3 In vitro pollen germination

The germination of the exposed and control pollen samples (4 batches from each condition) occurred at room temperature, in the dark, for 24 h, in an aqueous solution (Dafni 1992), using an optimized culture media (1% Saccharose, 0.6% polyethylene glycol 20 kDa and 2.5 mM $MgSO_4$, 8.3 mM $Ca(NO_3)_2$, 1.6 mM H_3BO_3, 1 mM KNO_3). At the end of the experiment, the samples were transferred to a slide, with a droplet of methylene blue. 200 pollen grains per slide were randomly counted using a light microscope (Olympus BX43, 400x) and the results were expressed as a percentage of germination. A pollen grain was

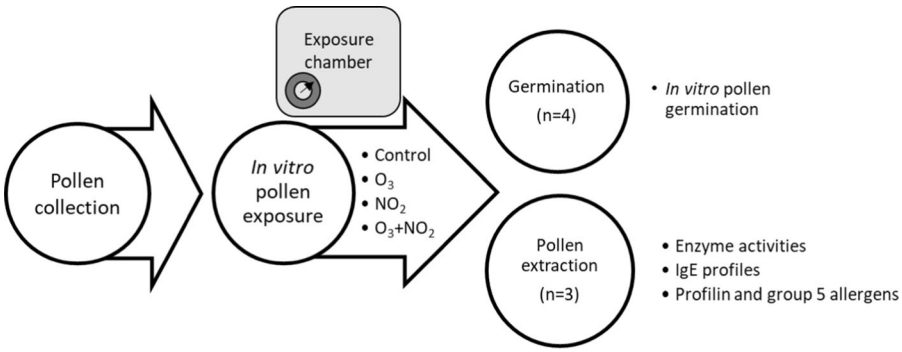

Fig. 1 Scheme of the experimental design

classified as germinated when the pollen tube was longer than the pollen size. Pollen tubes were classified as *long* and *short* when its size was higher or lower than twice the diameter of pollen, respectively.

2.4 Preparation of pollen extracts and total protein quantification

Pollen extracts from control or exposed to pollutants (O_3, NO_2 or $O_3 + NO_2$) (3 per treatment) were prepared in phosphate buffered (80 mM Na_2HPO_4, 20 mM de KH_2PO_4, pH = 7.8). Pollen grains were macerated (2.5 mg pollen/ml) in precooled buffer (4 °C) in a mortar. The pollen suspensions were then centrifuged at 10,000 *g* for 10 min, and the supernatants were recovered and frozen until analysis.

The soluble protein concentration in the supernatants was measured using the dye-binding Bradford protein assay (Bradford 1976), using bovine serum albumin as the standard.

2.5 SOD, catalase and GPx enzymatic activities

Total SOD (EC 1.15.1.1) activity quantification was based on the inhibition of the riboflavin-induced reduction of nitro blue tetrazolium (NBT), since superoxide anion is scavenged by SOD. The assay solution (pH = 7.8) contained 70 mM phosphate buffer, 10 µM EDTA, and 0,003% Triton X-100, 2 µM riboflavin and 10 µM NBT. NBT reduction was triggered by cool white fluorescent light (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and reduced NBT was measured at 560 nm, every 5 min, for 40 min. Reduction rates obtained in the presence (v) or absence (V) of pollen extract were used to calculate SOD enzymatic activity (U) as 1-(v/V), according to (Beauchamp and Fridovich 1971). Specific activity was expressed as U per mg of protein.

Catalase (EC 1.11.1.6) activity was determined as previously described by (Bailly et al. 2004) by spectrophotometrically following H_2O_2 consumption at 240 nm. Assay solution (pH = 7) contained 50 mM phosphate buffer and 10 mM H_2O_2. The results were expressed as specific activity, i.e. as µmol H_2O_2 decomposed/min/mg of protein.

The GPx (EC 1.11.1.9) activity was measured using *t*-butyl hydroperoxide (tBH) and reduced glutathione (GSH) as substrates, and the product, oxidized glutathione (GSSG), is then recycled back to GSH, employing glutathione reductase (GR) and NADPH, in a coupled reaction (assay solution, pH = 7.2: phosphate buffer 50 mM, 2.5 mM GSH, 0.5 mM NaN_3, 0.3 mM EDTA, 0.1 mM NADPH, 0.5 U GR, 0.4 mM tBH). Enzymatic activity of GPx is expressed as NADPH consumption, at 320 nm, µmol/min/mg of protein (Mannervik 1985).

Enzyme activity was assessed in all the pollen extracts prepared (3 per treatment). Technical replicates (4–5) were performed. For each extract, the enzyme activity was estimated as the mean of the technical replicates.

2.6 IgE-profiles and immunoblotting

Protein pollen extracts from the different samples (50 µg/lane) were separated in 12% polyacrylamide gels under reducing conditions (Laemmli 1970). For immunoblotting analysis, the proteins were electroblotted onto PVDF membrane (Westran, Whatman™, GE Healthcare). The membranes were saturated during 2 h in a blocking solution (5% non-fat dry milk (w/v) prepared in Tris–HCl buffered saline solution with Tween (TBST, pH = 7.6: 25 mM Tris–HCl, 150 mM NaCl and 0.1% Tween).

For IgE profiles, the membranes were incubated overnight (OV) at 4 °C with pooled sera from non-sensitized and sensitized patients to *D. glomerata* pollen diluted 1:20 in TBST. This study was performed under the Portuguese laws for Personal Data protection, DL 67/98 and DL 12/2005 and authorization GD/44721/2015 of the Ethics Committee for Health Science Research of the University of Évora; participant's signed informed consent forms before enrolment in the study. Bound specific IgE was detected with mouse anti-human IgE antibody (I6510, Sigma) diluted 1:10.000, incubated for 2 h, at room temperature (RT) and subsequently by a rabbit anti-mouse IgG horseradish peroxidase (HRP) conjugate (A9044, Sigma), diluted 1:30.000 and incubated for 2 h, RT. Colorimetric revelation was performed with TMB (T0565, Sigma), and a photographic image was taken.

For the identification of grasses group 5 allergen, biotinylated anti-Phl p5 monoclonal antibody (Allergopharma Joaquim Ganzer KG), 1:100, OV, 4 °C, was used, followed by streptavidin-peroxidase (S5512—Sigma) diluted 1:250, for 2 h, at RT. TMB colorimetric revelation was performed. Profilin was detected

using a rabbit anti-profilin antibody (kindly provided by Probelte Pharma, 500x diluted), OV, at 4 °C, followed by a second detection step using an anti-rabbit IgG-alkaline phosphatase conjugate (NIF1317, GE Healthcare), incubated for 2 h, at RT; fluorescent substrate (RPN5785, GE Healthcare) was used for revelation. Image was acquired using Gel-Doc (Bio-RAD) under UV light. Two independent experiments were performed for each evaluation.

Image processing, involving spatial and density measurements, were performed using the software *ImageJ* (https://imagej.nih.gov/ij/index.html). Only bands presenting density variation above 10% were considered different from the control.

2.7 Data analysis

The results were expressed as mean ± standard error of the mean (SEM) unless stated otherwise in the figure legends. Data normality was confirmed by Kolmogorov–Smirnov test and QQ-plots and homogeneity of variance was assessed by Levene test. Statistical assessment of differences between mean values was performed using One-Way ANOVA ($p < 0.1$ or $p < 0.05$ were accepted) (Zar 2014). SPSS was used for statistical analysis (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp, IBM Corp. Released 2017).

Graphs were produced using OriginPro software (OriginLab corporation).

3 Results

The *D. glomerata* pollen in vitro germination presented rates from 3.6 to 11.3%. Pollen germination capacity was threefold increased by NO_2 exposure and was not significantly affected by O_3 or $NO_2 + O_3$ (Fig. 2a). When the length of the pollen tubes was taken into consideration, the percentage of germinated pollen with long tubes was twofold higher in pollen exposed to $NO_2 + O_3$ (Fig. 2b).

Enzymatic activities of ROS scavenging enzymes are presented in Fig. 3.

The SOD activity was 1.7-fold higher and 2.2-fold higher in pollen exposed to NO_2 ($p < 0.1$) or to $NO_2 + O_3$ ($p < 0.05$) compared to controls (280 ± 56, 463 ± 69 and 604 ± 102 U/mg protein, for control, NO_2 and $NO_2 + O_3$ groups, respectively). O_3 alone did not significantly affect SOD activity (Fig. 3a).

The catalase activity was 0.06 ± 0.02 µmol H_2O_2/min/mg protein in the controls and was unaffected by NO_2 exposure. In the contrary, the catalase activity was enhanced, respectively twofold and fivefold in pollen exposed to O_3 (0.13 ± 0.05 µmol H_2O_2/min/mg protein; $p < 0.1$) or to $NO_2 + O_3$ (0.33 ± 0.12 µmol H_2O_2/min/mg protein; $p < 0.1$) (Fig. 3b).

The GPx activity was similar among groups ($6.9 \times 10^{-5} \pm 4.4 \times 10^{-5}$, $9.2 \times 10^{-5} \pm 3.6 \times 10^{-5}$, $9.2 \times 10^{-5} \pm 5.4 \times 10^{-5}$ and $10.7 \times 10^{-5} \pm 7.4 \times 10^{-5}$ µmol NADPH/min/mg protein, for

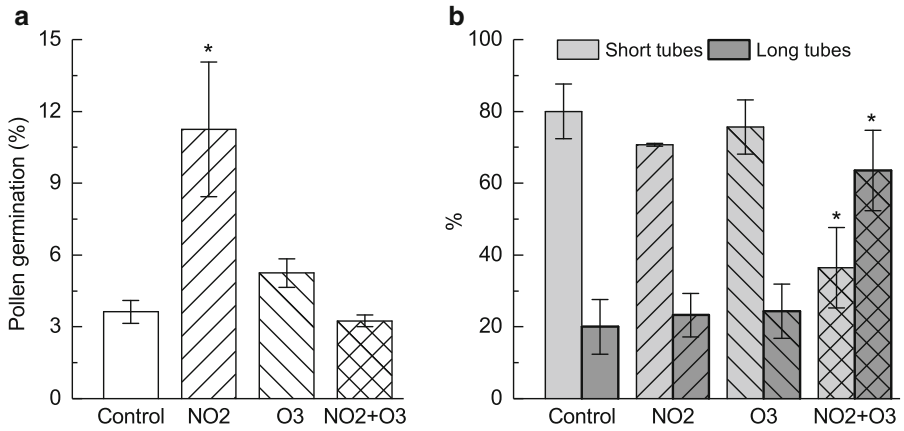

Fig. 2 Pollen germination in vitro. **a** Percentage of germination; **b** Percentage of short (1–2*Dp) and long (> 2*Dp) tubes of germinated pollen. 4 batches of pollen from each treatment were incubated in germination media. Each column represents the mean ± SEM. Statistical significance was assessed by One-Way ANOVA (*$p < 0.05$, relatively to control). Dp = Diameter of the pollen grain. See Appendix A, Fig. A1, for supplementary information

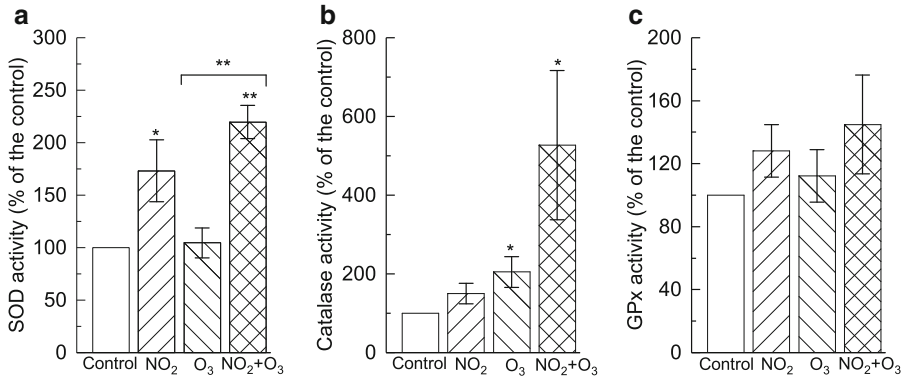

Fig. 3 ROS scavenging enzyme activities: **a** SOD (inhibition of the riboflavin-induced reduction of NBT—U/mg); **b** Catalase (consumption of H_2O_2/min/mg protein); **c** GPx (consumption of NADPH/min/mg protein). Extracts were prepared from batches of pollen (3 per treatment). To determine the enzyme activity for each pollen extract, 3–4 technical replicates were performed and the enzyme activity was estimated as the mean value of the technical replicates. Each column represents the mean ± SEM of the estimated activity for the 3 different extracts per treatment. Statistical significance was accessed by One-Way ANOVA (*$p < 0.1$, **$p < 0.05$)

control, NO_2, O_3 and $NO_2 + O_3$ groups, respectively) (Fig. 3c).

Protein bands, using SDS_PAGE, were observed ranging 10–90 kDa. The IgE reactivity profile of *D. glomerata* pollen, control and the IgE reactivity profile of *D. glomerata* pollen, control and exposed to pollutants, was investigated using a pool of sera from grass-sensitized patients (Fig. 4a). Several protein bands with similar molecular weight (MW) were identified in all groups (range 14.2–88.1 kDa). The bands with MW 29.0 ± 0.9, 33.5 ± 0.5, 41.7 ± 0.4, 49.6 ± 0.3, 53.9 ± 0.5, 56.6 ± 0.5 and 61.6 ± 0.7 kDa presented the highest density (≥ 5%) (Fig. 4a). A negative control, using a pool of sera of IgE-negative patients, was also performed, in the same conditions, and no bands were detected (data not shown).

The treatments differentially affected the band density (% of control; Fig. 4b); The bands with MW

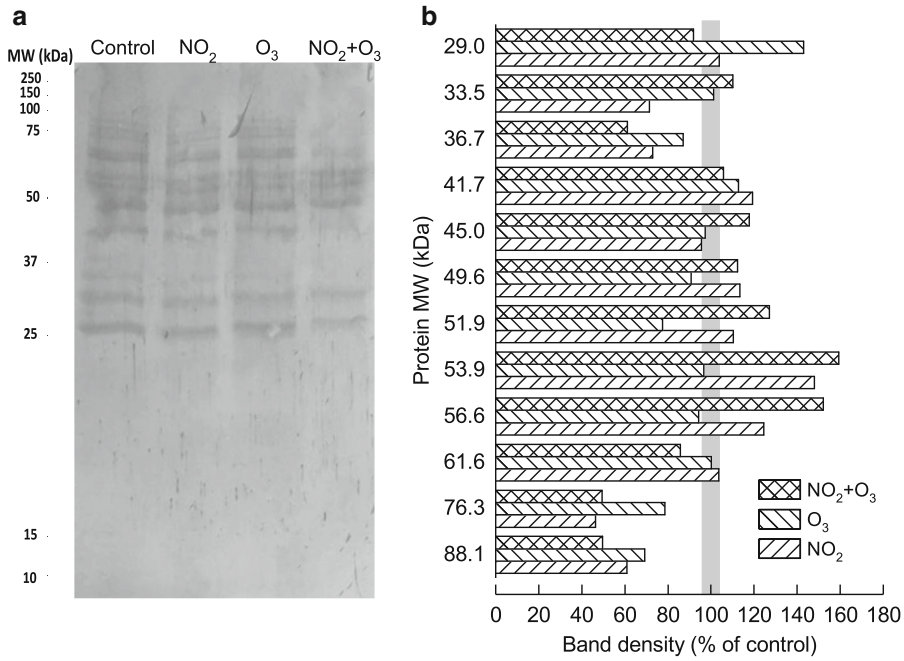

Fig. 4 Exposure of *D. glomerata* pollen to atmospheric pollutants induced changes in allergenic profile. **a** IgE profile (representative of two independent experiments); **b** Relative Density (% of control) of the IgE reactive bands (cut off 2%). See Appendix B, Table B1, for supplementary information. See Appendix A, Fig. A2, and Appendix B, table B1, for supplementary information

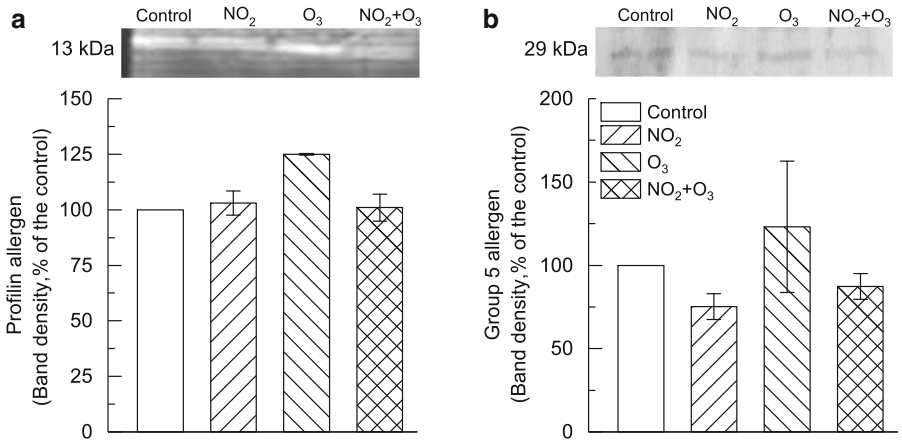

Fig. 5 Immunoblots and relative density (% of control) for profilin (**a**) and group 5 allergen (**b**). Specific IgG antibodies were used. Data represent the analysis of two independent experiments

41.7, 45.0, 49.6, 51.9, 53.9 and 56.6 kDa from NO_2 treated pollen presented augmented relative density (11–49%) while the bands with MW 36.7, 76.3 and 88.1 kDa presented diminished relative density (29–55%).

O_3 exposed pollen showed an enhancement of the bands with MW 29.0, 31.0 and 41.7 kDa (42%, 50% and 13%, respectively) and a reduction of the relative density of bands with MW 36.7, 51.9, 76.3 and 88.1 kDa (21–32%).

Finally, the bands with MW 45, 46,7, 51,9, 53.9 and 56.6 kDa were intensified (13–61%) by the exposure to both pollutants while the bands with MW 36.7, 61.6, 76.3 and 88.1 kDa were diminished by 15–52%.

The expression of profilin and grass group 5 allergens was assessed by immunoblot (Fig. 5). Profilin was identified as a peptide with 13 kDa and was induced 1.2-fold by O_3 treatment. Group-5 allergen presented a molecular mass of 29 kDa, which is compatible with the IgE-reactivity for the band 29.0 ± 0.9 kDa and was 1.6-fold induced by O_3 exposure.

4 Discussion

In this work it is shown that the pollutants NO_2 and O_3, which can be derived from vehicle traffic emissions, evoked differentiated effects on *D. glomerata* pollen. Differential effects caused by these agents in other pollen types have been described in multiple studies, either after in vitro exposure (Cuinica et al. 2014; Lu et al. 2014; Ribeiro et al. 2014; Sousa et al. 2012), as in this work, or during environmental exposure (Cortegano et al. 2004; Ghiani et al. 2012; Gottardini et al. 2004).

Starting with an overview of the most relevant results obtained in each test condition and our interpretation based on current knowledge, we seek to discuss the real-world implications of pollen exposure to traffic related pollutants.

4.1 Effects of pollen exposure to NO_2

Exposure to NO_2 induced a threefold increase in the germination rate of *D. glomerata* pollen, despite the low germination rate observed probably related with the storage conditions of the pollen samples since Cauneau-Pigot (1991) showed for this pollen species a reduction in germination after storage at − 20 °C compared with the fresh pollen.

A contradictory effect of NO_2 was observed in pollen germination from *Betula pendula* R., *Ostrya carpinifolia* S. and *Carpinus betulus* L. (Cuinica et al. 2014) and from *Acer negundo* L. pollen (Sousa et al. 2012) after NO_2 fumigation. Structural and/or biochemical characteristics of different pollen types may explain this opposing response. Indeed, at the seed germination level, for instance, the effects of NO_2 are also inconsistent where both impairment or promotion of germination have been described, depending on the species analysed (Beligni and Lamattina 2000; Keeley and Fotheringham 1997).

It has been shown that the uptake of NO_2 by *Phleum pratense* L. pollen is potentiated by water content, inducing a subsequent acidification (Chassard et al. 2015), as a result of NO_2^- (Bright et al. 2009; Chassard et al. 2015) and NO_3^- (Chassard et al. 2015)

formation. Additionally, NO_2^- promoted synthesis of intracellular NO, an important signalling molecule in the process of pollen germination (Pasqualini et al. 2015). NO synthesis, favoured by NO_2 exposure, could explain the increase in the germination rate observed in this work.

At the level of the scavenging enzymes of ROS, catalase and GPx, there were no significant changes induced by NO_2. However, SOD activity recorded after exposure to NO_2 was higher than the control suggesting some oxidative stress in pollen, driven by superoxide anion, generated by NO_2 exposure. The activity of the catalase necessary to eliminate the H_2O_2 generated by cell metabolism, or by the action of SOD, consistently presented an average activity above the control. The physiological levels of ROS play important roles in germination signalling (Pasqualini et al. 2015), thus the ROS imbalance suggested by enzymatic activities measured in this work may partially explain the increase in germination rate observed.

Proteomic shifts have been observed previously in pollen exposed to pollutants, possibly affecting its allergenic potential. For instance, a higher IgE recognition of some protein bands after NO_2 fumigation (in vitro, 0.039 and 0.065 ppm, 6 h) was described for *B. pendula*, *O. carpinifolia and C. betulus* pollen (Cuinica et al. 2014) while a diminished IgE binding to some allergens (Phl p 2, Phl p 5b and Phl p 6) was described after *P. pratensis* pollen exposure to NO_2 (in vitro, 2 ppm, 2 h) (Rogerieux et al. 2007).

In our experiments, pollen exposure to NO_2 modified the immune reactivity of several proteins, five became over- and four became under-recognized. Among the proteins showing decreased immunoreactivity is a band of molecular weight 33.5 kDa, compatible with the grass group 1 major allergen (Kleine-Tebbe 2014). The grass group 5 major allergen is either unchanged or slightly diminished while five minor allergens with MW ranging 41–56.6 kDa that may include Group 4 and Group 13 are increased. Moreover, SOD activity, an allergen with MW 23 kDa recently described in *P. pratense* (Conti et al. 2014) is elevated in pollen exposed to NO_2. Whilst not ruled out, when taken together it is unclear whether the pollutant NO_2 induces a significant increase in *D. glomerata* pollen allergenicity.

4.2 Effects of pollen exposure to O_3

The exposure of the pollen to O_3 did not affect the germination rate and pollen tubes length as both were not significantly different to the control values. However, the duplication of catalase activity in pollen exposed to O_3, denotes oxidative stress generated by this gas, leading to increased production of H_2O_2 (Pessarakli et al. 2019). Since SOD activity was not changed by the exposure to this pollutant, the H_2O_2 might have been generated by a SOD-independent mechanism, possibly the induction of NAD(P)H oxidase, a mechanism described by Pasqualini et al. (2011) for *Ambrosia artemisiifolia* pollen exposed to O_3. GPx activity did not significantly change, suggesting that GSH-dependent pathway was not preferentially used to deal with O_3-induced oxidative stress.

At the level of allergen expression, there were several changes to the IgE-recognition pattern, with diminished recognition of four proteins (MW of 36.7, 51.9, 76.3 and 88.1 kDa) and increased recognition in three proteins (MW of 29, 31 and 13 kDa); two of the latter were identified by Western blot as grass group 5 (EACCI 2014) and profilin (grass Group 12), a pan-allergen presenting high homology between plant cells, responsible for cross-reactivity between pollen types (Hauser et al. 2010). Diminished IgE-binding to Phl p 5b have been reported by Rogerieux and colleagues (Rogerieux et al. 2007) probably resulting from different pollen exposure protocols; in fact, the kinetics and dose-response of the effects of pollen exposures to O_3 are yet unknown.

O_3 seems to cause an augmented allergenicity of grass pollen, characterized by the increased immune recognition of one of its major allergens and a pan-allergen that may be responsible for important cross-reactivity thus aggravating allergic symptomatology.

4.3 Effects of pollen exposure to both to NO_2 and O_3

Pollen germination rate was unaffected by simultaneous exposure to both pollutants. Nevertheless, pollen tubes of the germinated grains were longer, revealing cell signalling changes favouring the process of pollen tube formation.

The increased enzymatic activities of SOD and catalase enzymes suggest oxidative stress, resulting from additive effects of both pollutants, with positive

effects on the pollen tube growth driven by reactive oxygen species (Pasqualini et al. 2015) without significantly affecting the germination. Due to a synergistic effect, the presence of O_3 increases NO_2 absorption (Chassard et al. 2015) and strongly favours the nitration of proteins (Franze et al. 2005) thus generating amplified SOD and catalase activity and altered IgE recognition patterns compared to O_3 or NO_2 alone. Indeed, in the IgE recognition profile six bands were intensified (MW in the range 41.7–56.6 kDa) and three have shown lower IgE-reactivity (MW of 36.7, 76.3 and 88.1 kDa). It is noteworthy that these bands correspond to the same ones identified in pollen fumigated with NO_2 alone and that the effect of NO_2 was amplified by the presence of O_3, either for increased or for decreased immune reactivities. Additionally, in accordance to NO_2 treatment, the increased SOD activity suggests an increase of the SOD allergen (Conti et al. 2014), compatible with a low intensity band of MW 22.6 ± 0.2 kDa detected in our allergograms.

As for NO_2 treatment, the major allergen and the pan-allergen profilin were unchanged thus the effect over allergenicity remains unclear.

4.4 An integrated overview

This work suggests that common air pollutants evoke a modified pollen allergenicity, potentially contributing to a higher incidence of respiratory allergic diseases in urban areas (D'Amato et al. 2016) with high vehicle traffic emissions.

Our results have shown that pollen oxidative defences are activated by common air pollutants, affecting both its germination capacity and its allergenic activity. Concerning the pollen germination, it is remarkable that NO_2 alone stimulated the germination, resulting from NO signalling (Pasqualini et al. 2015), while O_3 did not affect germination. On the other hand, $NO_2 + O_3$ induced the formation of longer pollen tubes, possibly due to increased ROS formation (Pasqualini et al. 2015), without affecting germination; in this condition, ROS formation might be highly favoured due to synergistic effect of the pollutants together, as suggest the increased SOD and catalase activities, and possibly lower NO formation compared to NO_2 alone, due to the skewing of the equilibrium to the protein nitration (Ghiani et al. 2012; Gruijthuijsen et al. 2006). From an allergenic point of view, when comparing the effects of NO_2 with the effect of NO_2 and O_3 together, an amplification of the NO_2 effect was observed. Additionally, the O_3 stimuli, separately, seems to be more harmful, at least in the case of grass species, as shown by our results. In fact, differential allergenic potency of airborne grass and olive pollen depending on the year and geographical location has been reported (Buters et al. 2015; Galan et al. 2013). The results shown in this work may contribute to explain these differences.

Modified immunoreactivity may be due to either a change in protein expression levels in response to the induced stress or to a change in antigen–antibody recognition patterns as result of chemical modification such as nitration of proteins epitopes (Gruijthuijsen et al. 2006), or by the combination of both aspects. Subsequent studies of post-translational modifications of proteins will be necessary to distinguish the contribution of each of these factors to the changes shown in this and other works. However, regardless of the pathway underlying immunoreactivity modification, the amplification of pollen allergenicity is expected to induce increased allergic reactions and aggravation of allergic symptoms.

Considering the elevated values of O_3 reached in the Alentejo region during the main grass pollen season (end of April to beginning of June) (Bortoli et al. 2009), an increased allergenic risk is expected, particularly if associated with high-vehicle traffic environments.

Acknowledgements This work was funded by the European Union through the European Regional Development Fund, framed in COMPETE 2020 (Operational Programme Competitiveness and Internationalization), through the ICT project (UID/GEO/04683/2013) with reference POCI-01-0145-FEDER-007690, and by National Funds by the Portuguese Foundation for Science and Technology (FCT) through the projects PTDC/ATP-EAM/0817/2014 and UIDB/04683/2020—ICT (Institute of Earth Sciences). The authors thank Russell Alpizar-Jara, CIMA (Research Centre for Mathematics and Applications) and Department of Mathematics, School of Science and Technology, University of Évora, for reviewing the statistical analysis.

References

Bailey-Serres, J., & Mittler, R. (2006). The roles of reactive oxygen species in plant cells. *Plant Physiology, 141*(2), 311. https://doi.org/10.1104/pp.104.900191

Bailly, C., Leymarie, J., Lehner, A., Rousseau, S., Come, D., & Corbineau, F. (2004). Catalase activity and expression in developing sunflower seeds as related to drying. *Journal of Experimental Botany, 55*(396), 475–483. https://doi.org/10.1093/jxb/erh050

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical biochemistry*, *44*(1), 276–87. https://www.ncbi.nlm.nih.gov/pubmed/4943714. Accessed 7 Apr 2017

Beligni, M. V., & Lamattina, L. (2000). Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta, 210*(2), 215–221. https://doi.org/10.1007/PL00008128

Bortoli, D., Silva, A. M., Costa, M. J., Domingues, A. F., & Giovanelli, G. (2009). Monitoring of atmospheric ozone and nitrogen dioxide over the south of Portugal by ground-based and satellite observations. *Optics Express, 17*(15), 12944. https://doi.org/10.1364/oe.17.012944

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. https://doi.org/10.1016/0003-2697(76)90527-3

Bright, J., Hiscock, S. J., James, P. E., & Hancock, J. T. (2009). Pollen generates nitric oxide and nitrite: a possible link to pollen-induced allergic responses. *Plant Physiology and Biochemistry, 47*(1), 49–55. https://doi.org/10.1016/j.plaphy.2008.09.005

Buters, J., Prank, M., Sofiev, M., Pusch, G., Albertini, R., Annesi-Maesano, I., et al. (2015). Variation of the group 5 grass pollen allergen content of airborne pollen in relation to geographic location and time in season the HIALINE working group. *Journal of Allergy and Clinical Immunology, 136*(1), 87-95.e6. https://doi.org/10.1016/j.jaci.2015.01.049

Cauneau-Pigot, A. (1991). Effects of freeze-drying upon in vitro germination and the mineral composition of allergenic dactylis glomerata pollen. *Grana, 30*(2), 547–552. https://doi.org/10.1080/00173139109432024

Chassard, G., Choël, M., Gosselin, S., Vorng, H., Petitprez, D., Shahali, Y., et al. (2015). Kinetic of NO_2 uptake by *Phleum pratense* pollen: Chemical and allergenic implications. *Environmental Pollution, 196,* 107–113. https://doi.org/10.1016/j.envpol.2014.10.004

Chehregani, A., Majde, A., Moin, M., Gholami, M., Ali Shariatzadeh, M., & Nassiri, H. (2004). Increasing allergy potency of *Zinnia* pollen grains in polluted areas. *Ecotoxicology and Environmental Safety, 58*(2), 267–272. https://doi.org/10.1016/J.ECOENV.2003.12.004

Conti, A., Alessio, M., Pesca, M., Soldarini, A., Breda, D., Pigatto, P. D., & Burastero, S. E. (2014). Phleum pratense manganese superoxide dismutase identified by proteomic: A new candidate grass allergen. *Annals of Allergy, Asthma and Immunology*. https://doi.org/10.1016/j.anai.2013.12.015

Cortegano, I., Civantos, E., Aceituno, E., Del Moral, A., López, E., Lombardero, M., et al. (2004). Cloning and expression of a major allergen from *Cupressus arizonica* pollen, Cup a 3, a PR-5 protein expressed under polluted environment. *Allergy: European Journal of Allergy and Clinical Immunology., 59*(5), 485–490. https://doi.org/10.1046/j.1398-9995.2003.00363.x

Cuinica, L. G., Abreu, I., & Esteves da Silva, J. (2014). Effect of air pollutant NO_2 on *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen fertility and human allergenicity. *Environmental Pollution, 186,* 50–55. https://doi.org/10.1016/J.ENVPOL.2013.12.001

D'Amato, G., Pawankar, R., Vitale, C., Lanza, M., Molino, A., Stanziola, A., et al. (2016). Climate change and air pollution: effects on respiratory allergy. *Allergy, Asthma & Immunology Research, 8*(5), 391–395. https://doi.org/10.4168/aair.2016.8.5.391

Dafni, A. (1992). Pollination ecology: a practical approach. In D. Rickwood & B. D. Hames (Eds.), *Pollination ecology: a practical approach*. Oxford: Oxford University Press.

EACCI. (2014). *Global atlas of allergy*. https://www.eaaci.org/GlobalAtlas/GlobalAtlasAllergy.pdf. Accessed 16 Jan 2020.

Franze, T., Weller, M. G., Niessner, R., & Pöschl, U. (2005). Protein Nitration by polluted air. *Environmental Science & Technology, 39*(6), 1673–1678. https://doi.org/10.1021/ES0488737

Galan, C., Antunes, C., Brandao, R., Torres, C., Garcia-Mozo, H., Caeiro, E., et al. (2013). Airborne olive pollen counts are not representative of exposure to the major olive allergen Ole e 1. *Allergy, 68*(6), 809–812. https://doi.org/10.1111/all.12144

Ghiani, A., Aina, R., Asero, R., Bellotto, E., & Citterio, S. (2012). Ragweed pollen collected along high-traffic roads shows a higher allergenicity than pollen sampled in vegetated areas. *Allergy, 67*(7), 887–894. https://doi.org/10.1111/j.1398-9995.2012.02846.x

Ghiani, A., Bruschi, M., Citterio, S., Bolzacchini, E., Ferrero, L., Sangiorgi, G., et al. (2016). Nitration of pollen aeroallergens by nitrate ion in conditions simulating the liquid water phase of atmospheric particles. *Science of the Total Environment, 573,* 1589–1597. https://doi.org/10.1016/j.scitotenv.2016.09.041

Gottardini, E., Cristofolini, F., Paoletti, E., Lazzeri, P., & Pepponi, G. (2004). Pollen viability for air pollution biomonitoring. *Journal of Atmospheric Chemistry, 49*(1–3), 149–159. https://doi.org/10.1007/s10874-004-1221-z

Gruijthuijsen, Y. K., Grieshuber, I., Stöcklinger, A., Tischler, U., Fehrenbach, T., Weller, M. G., et al. (2006). Nitration enhances the allergenic potential of proteins. *International Archives of Allergy and Immunology, 141*(3), 265–275. https://doi.org/10.1159/000095296

Hauser, M., Roulias, A., Ferreira, F., & Egger, M. (2010). Panallergens and their impact on the allergic patient. *Allergy, Asthma and Clinical Immunology*. https://doi.org/10.1186/1710-1492-6-1

Iriti, M., & Faoro, F. (2007). Oxidative stress, the paradigm of ozone toxicity in plants and animals. *Water Air and Soil Pollution, 187*(1–4), 285–301. https://doi.org/10.1007/s11270-007-9517-7

Keeley, J. E., & Fotheringham, C. J. (1997). Trace gas emissions and smoke-induced seed germination. *Science, 276*(5316), 1248–1250. https://doi.org/10.1126/science.276.5316.1248

Kleine-Tebbe, J. (2014). Grass pollen allergens. In I. Editors Akdis, C.A., & Agache (Ed.), *Global atlas of allergy* (p.

22). European Academy of Allergy and Clinical Immunology Publishers.

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685. https://doi.org/10.1038/227680a0

Lu, S., Ren, J., Hao, X., Liu, D., Zhang, R., Wu, M., et al. (2014). Characterization of protein expression of *Platanus* pollen following exposure to gaseous pollutants and vehicle exhaust particles. *Aerobiologia, 30*(3), 281–291. https://doi.org/10.1007/s10453-014-9327-5

Mannervik, B. (1985). Glutathione peroxidase. *Methods in Enzymology, 113,* 490–495. https://doi.org/10.1016/s0076-6879(85)13063-6

Miranda, P., Coelho, F. E. S., Tomé, A. R., & Valente, M. A. (2001). 20th century Portuguese climate and climate scenarios. In F. D. Santos, K. Forbes, & R. Moita (Eds.), *Climate change in Portugal. Scenatios, impacts and adaptation measures—SIAM. Executive summary and conclusions* (pp. 23–84). Lisbon: Gradiva.

Monteiro, A., Miranda, A. I., Borrego, C., & Vautard, R. (2007). Air quality assessment for Portugal. *Science of The Total Environment, 373*(1), 22–31. https://doi.org/10.1016/J.SCITOTENV.2006.10.014

Pasqualini, S., Cresti, M., Del Casino, C., Faleri, C., Frenguelli, G., Tedeschini, E., & Ederli, L. (2015). Roles for NO and ROS signalling in pollen germination and pollen-tube elongation in *Cupressus arizonica*. *Biologia Plantarum, 59*(4), 735–744. https://doi.org/10.1007/s10535-015-0538-6

Pasqualini, S., Tedeschini, E., Frenguelli, G., Wopfner, N., Ferreira, F., D'Amato, G., & Ederli, L. (2011). Ozone affects pollen viability and NAD(P)H oxidase release from Ambrosia artemisiifolia pollen. *Environmental Pollution (Barking, Essex: 1987), 159*(10), 2823–30. https://doi.org/https://doi.org/10.1016/j.envpol.2011.05.003

Pessarakli, M., Kordrostami, M., Rabiei, B., & Ebadi, A. A. (2019). Oxidative stress in plants. In *Handbook of plant and crop stress, fourth edition* (pp. 85–92). Boca Raton: CRC Press. https://doi.org/https://doi.org/10.1201/9781351104609-6

Ribeiro, H., Duque, L., Sousa, R., & Abreu, I. (2013). Ozone effects on soluble protein content of *Acer negundo*, *Quercus robur* and *Platanus* spp. pollen. *Aerobiologia, 29*(3), 443–447. https://doi.org/10.1007/s10453-013-9291-5

Ribeiro, H., Duque, L., Sousa, R., Cruz, A., Gomes, C., Esteves da Silva, J., & Abreu, I. (2014). Changes in the IgE-reacting protein profiles of *Acer negundo*, *Platanus x acerifolia* and *Quercus robur* pollen in response to ozone treatment. *International Journal of Environmental Health Research, 24*(6), 515–527. https://doi.org/10.1080/09603123.2013.865716

Ring, J., Eberlein-Koenig, B., & Behrendt, H. (2001). Environmental pollution and allergy. *Annals of Allergy, Asthma & Immunology, 87*(6), 2–6. https://doi.org/10.1016/S1081-1206(10)62332-0

Rogerieux, F., Godfrin, D., Sénéchal, H., Motta, A. C., Marlière, M., Peltre, G., & Lacroix, G. (2007). Modifications of *Phleum pratense Grass* Pollen Allergens following artificial exposure to gaseous air pollutants (O_3, NO_2, SO_2). *International Archives of Allergy and Immunology, 143*(2), 127–134. https://doi.org/10.1159/000099079

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 SO_2 and NO_2: Effects on allergenicity and germination. *Environmental Science & Technology, 46*(4), 2406–2412. https://doi.org/10.1021/es2034685

Zar, J. H. (2014). *Biostatistical analysis* (5th ed.). New International: Pearson.

Zumdahl, S. (2009). Chemistry in the atmosphere. In H. Cheek (Ed.), *Chemical principles* (6th ed., pp. 176–180). Boston: Houghton Mifflin Company.

Appendix A:

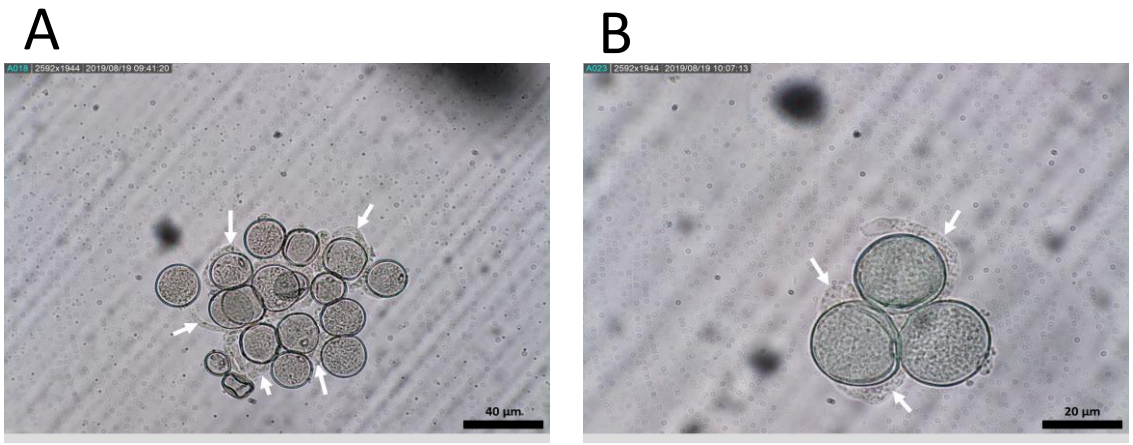

Figure A1: Microphotographs showing germinated pollen obtained at 200x (A) and 400x (B) magnification. Arrows indicate examples of pollen tubes that, in the conditions used, usually grows around the pollen grain.

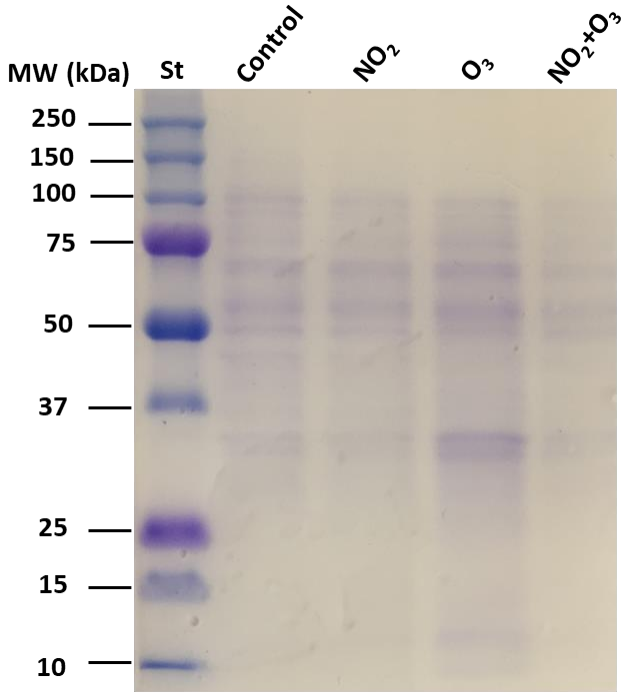

Figure A2: Coomassie-blue stained SDS-Page profiles. Protein pollen extracts from the different samples (~50 µg/lane) were separated in 12% polyacrylamide gels, as described in Methods.

Appendix B

Table B1: Molecular weights of the IgE profile (mean ± SD, n=2) and the relative densities (%) for each test group.

MW (kDa)	Control	NO_2	O_3	NO_2+O_3
88.1 ± 1.7	3	2	2	2
76.3 ± 6.6	3	<2	2	<2
61.6 ± 0.7	8	8	8	7
56.6 ± 0.5	6	7	5	8
53.9 ± 0.5	5	8	5	8
51.9 ± 0.4	3	3	2	2
49.6 ± 0.3	7	8	6	8
45.0 ± 0.5	2	2	2	3
41.7 ± 0.4	5	6	6	6
37.1 ± 0.8	2	<2	2	2
36.7 ± 0.0	3	2	2	2
33.5 ± 0.5	6	4	6	7
31.0 ± 1.1	<2	<2	2	<2
29.0 ± 0.9	8	9	12	8
26.5 ± 0.7	<2	<2	2	<2
22.6 ± 0.2	2	<2	<2	<2
19.7 ± 0.1	<2	<2	2	2
18.6 ± 0.3	<2	<2	2	<2
17.9 ± 0.1	<2	2	2	2
17.2 ± 0.2	<2	2	<2	2
15.9 ± 0.1	<2	<2	<2	2

Appendix C

Tables C1-C6: One-Way ANOVA tables of statistical analyses: SOD activity.

C1

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (SOD)	3	300	100	0
NO2 (SOD)	3	519,4535	173,1512	2619,107

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	8026,642	1	8026,642	6,129296	0,068523	7,708647
Within groups	5238,215	4	1309,554			
Total	13264,86	5				

C2

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (SOD)	3	300	100	0
O3 (SOD)	3	313,6301	104,5434	621,6134

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	30,96343	1	30,96343	0,099623	0,768065	7,708647
Within groups	1243,227	4	310,8067			
Total	1274,19	5				

C3

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (SOD)	3	300	100	0
NO2+O3 (SOD)	3	659,1791	219,7264	763,543

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	21501,61	1	21501,61	56,32063	0,001687	7,708647
Within groups	1527,086	4	381,7715			
Total	23028,7	5				

C4

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
O3 (SOD)	3	313,6301	104,5434	621,6134
NO2+O3 (SOD)	3	659,1791	219,7264	763,543

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	19900,69	1	19900,69	28,73421	0,005845	7,708647
Within groups	2770,313	4	692,5782			
Total	22671	5				

C5

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
NO2 (SOD)	3	519,4535	173,1512	2619,107
O3 (SOD)	3	313,6301	104,5434	621,6134

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	7060,545	1	7060,545	4,357392	0,105135	7,708647
Within groups	6481,441	4	1620,36			
Total	13541,99	5				

C6

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
NO2 (SOD)	3	519,4535	173,1512	2619,107
NO2+O3 (SOD)	3	659,1791	219,7264	763,543

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	3253,875	1	3253,875	1,923861	0,237717	7,708647
Within groups	6765,3	4	1691,325			
Total	10019,18	5				

Tables C7-C12: One-Way ANOVA tables of statistical analyses: Catalase (CAT) activity.

C7

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (CAT)	3	300	100	1,01E-28
NO2 (CAT)	3	449,5891	149,863	2094,262

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	3729,485	1	3729,485	3,561622	0,132173	7,708647
Within groups	4188,525	4	1047,131			
Total	7918,01	5				

C8

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (CAT)	3	300	100	1,01E-28
O3 (CAT)	3	614,3631	204,7877	4481,61

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	16470,69	1	16470,69	7,350346	0,053474	7,708647
Within groups	8963,22	4	2240,805			
Total	25433,91	5				

C9

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (CAT)	3	300	100	1,01E-28
NO2+O3 (CAT)	3	1581,903	527,3011	107659,5

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	273879,4	1	273879,4	5,087882	0,0871	7,708647
Within groups	215319	4	53829,74			
Total	489198,4	5				

C10

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
NO2 (CAT)	3	449,5891	149,863	2094,262
O3 (CAT)	3	614,3631	204,7877	4481,61

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	4525,076	1	4525,076	1,376266	0,305829	7,708647
Within groups	13151,75	4	3287,936			
Total	17676,82	5				

C11

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
NO2 (CAT)	3	449,5891	149,863	2094,262
NO2+O3 (CAT)	3	1581,903	527,3011	107659,5

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	213689,3	1	213689,3	3,893976	0,119714	7,708647
Within groups	219507,5	4	54876,88			
Total	433196,8	5				

C12

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
O3 (CAT)	3	614,3631	204,7877	4481,61
NO2+O3 (CAT)	3	1581,903	527,3011	107659,5

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	156022,4	1	156022,4	2,782608	0,170618	7,708647
Within groups	224282,2	4	56070,55			
Total	380304,6	5				

Tables C13-C18: One-Way ANOVA tables of statistical analyses: Glutathione Peroxidase (GPx) activity.

C13

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (GPx)	3	300	100	0
NO2 (GPx)	3	384,5302	128,1767	827,0267

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	1190,893	1	1190,893	2,879937	0,164927	7,708647
Within groups	1654,053	4	413,5134			
Total	2844,946	5				

C14

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (GPx)	3	300	100	0
O3 (GPx)	3	337,0355	112,3452	834,9361

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	228,6042	1	228,6042	0,547597	0,50038	7,708647
Within groups	1669,872	4	417,4681			
Total	1898,476	5				

C15

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control (GPx)	3	300	100	0
NO2+O3 (GPx)	3	434,6143	144,8714	2967,41

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	3020,17	1	3020,17	2,03556	0,226818	7,708647
Within groups	5934,82	4	1483,705			
Total	8954,99	5				

C16

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
NO2 (GPx)	3	384,5302	128,1767	827,0267
O3 (GPx)	3	337,0355	112,3452	834,9361

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	375,9585	1	375,9585	0,452427	0,538042	7,708647
Within groups	3323,926	4	830,9814			
Total	3699,884	5				

C17

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
NO2 (GPx)	3	384,5302	128,1767	827,0267
NO2+O3 (GPx)	3	434,6143	144,8714	2967,41

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	418,0701	1	418,0701	0,22036	0,663212	7,708647
Within groups	7588,873	4	1897,218			
Total	8006,943	5				

C18

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
O3 (GPx)	3	337,0355	112,3452	834,9361
NO2+O3 (GPx)	3	434,6143	144,8714	2967,41

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	1586,94	1	1586,94	0,834716	0,412601	7,708647
Within groups	7604,692	4	1901,173			
Total	9191,632	5				

Tables C19-C24: One-Way ANOVA tables of statistical analyses: Pollen germination.

C19

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control pollen	4	14,5	3,625	0,895833
NO2 pollen	4	45	11,25	31,75

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	116,2813	1	116,2813	7,123803	0,037074	5,987378
Within groups	97,9375	6	16,32292			
Total	214,2188	7				

C20

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
NO2 pollen	4	45	11,25	31,75
O3 pollen	4	21	5,25	1,416667

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	72	1	72	4,341709	0,082315	5,987378
Within groups	99,5	6	16,58333			
Total	171,5	7				

C21

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control pollen	4	14,5	3,625	0,895833
O3 pollen	4	21	5,25	1,416667

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	5,28125	1	5,28125	4,567568	0,076447	5,987378
Within groups	6,9375	6	1,15625			
Total	12,21875	7				

C22

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
NO2 pollen	4	45	11,25	31,75
NO2+O3 pollen	4	13	3,25	0,25

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	128	1	128	8	0,03002	5,987378
Within groups	96	6	16			
Total	224	7				

C23

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control pollen	4	14,5	3,625	0,895833
NO2+O3 pollen	4	13	3,25	0,25

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	0,28125	1	0,28125	0,490909	0,509766	5,987378
Within groups	3,4375	6	0,572917			
Total	3,71875	7				

C24

Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
O3 pollen	4	21	5,25	1,416667
NO2+O3 pollen	4	13	3,25	0,25

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	8	1	8	9,6	0,02116	5,987378
Within groups	5	6	0,833333			
Total	13	7				

Tables C25-C30: One-Way ANOVA tables of statistical analyses: Tube length.

C25: Long Tubes
Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control	4	80	20	229,6296
NO2	4	93,1114	23,27785	146,7479

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	21,4886	1	21,4886	0,114186	0,746937	5,987378
Within groups	1129,133	6	188,1888			
Total	1150,621	7				

C26: Short tubes
Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control	4	320	80	229,6296
NO2	3	212,0168	70,67227	0,5155

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	149,1541	1	149,1541	1,080952	0,346124	6,607891
Within groups	689,9199	5	137,984			
Total	839,074	6				

C27: Long Tubes
Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control	4	80	20	229,6296
O3	4	97,54274	24,38568	226,4621

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	38,46844	1	38,46844	0,168687	0,695546	5,987378
Within groups	1368,275	6	228,0458			
Total	1406,743	7				

C28: Short tubes
Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control	4	320	80	229,6296
O3	4	302,4573	75,61432	226,4621

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	38,46844	1	38,46844	0,168687	0,695546	5,987378
Within groups	1368,275	6	228,0458			
Total	1406,743	7				

C29: Long Tubes
Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control	4	80	20	229,6296
NO2+O3	4	254,1667	63,54167	502,0255

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	3791,753	1	3791,753	10,36487	0,01815	5,987378
Within groups	2194,965	6	365,8275			
Total	5986,719	7				

C30: Short tubes
Anova: One-Way

SUMMARY

Groups	*N*	*Sum*	*Mean*	*Variance*
Control	4	320	80	229,6296
NO2+O3	4	145,8333	36,45833	502,0255

ANOVA Table

Source	*SQ*	*fl*	*MQ*	*F*	*P value*	*F critical*
Between groups	3791,753	1	3791,753	10,36487	0,01815	5,987378
Within groups	2194,965	6	365,8275			
Total	5986,719	7				