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In Vitro Exposure of *Acer negundo* Pollen to Atmospheric Levels of SO_2 and NO_2: Effects on Allergenicity and Germination

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ABSTRACT: In the last years, a rising trend of pollen allergies in urban areas has been attributed to atmospheric pollution. In this work, we investigated the effects of SO_2 and NO_2 on the protein content, allergenicity, and germination rate of *Acer negundo* pollen. A novel environmental chamber was assembled to exposure pollen samples with SO_2 or NO_2 at two different levels: just below and two times the atmospheric hour-limit value acceptable for human health protection in Europe. Results showed that protein content was lower in SO_2-exposed pollen samples and slightly higher in NO_2-exposed pollen compared to the control sample. No different polypeptide profiles were revealed by SDS-PAGE between exposed and nonexposed pollen, but the immunodetection assays indicated higher IgE recognition by all sera of sensitized patients to *Acer negundo* pollen extracts in all exposed samples in comparison to the nonexposed samples. A decrease in the germination rate of exposed in contrast to nonexposed pollen was verified, which was more pronounced for NO_2-exposed samples. Our results indicated that in urban areas, concentrations of SO_2 and NO_2 below the limits established for human protection can indirectly aggravate pollen allergy on predisposed individuals and affect plant reproduction.

INTRODUCTION

Pollen grains are biological structures produced by superior plants to perform the vital task of sexual reproduction. For successful fertilization, anemophilous plants developed compensatory mechanisms, such as the release of large amounts of airborne pollen and production of aerodynamic pollen grains, making dispersal easier. These features, associated with the allergens present in both inner part of the pollen wall (intine and cytoplasm) and pollen outer wall (submicroscopical sites of the exine and orbicules) (reviewed in ref 1) make these aerosol biological particles triggers of respiratory allergic reactions. In the past few decades, a wealth of environmental and epidemiological studies has highlighted a relation between air pollutants and pollen on the exacerbation of respiratory allergy symptoms in most urbanized societies.[2−5] Although the role of atmospheric pollutants on the allergic sensitivity of airways is not yet completely clear, it has been suggested that exposure to high levels of pollutants such as nitrogen oxides (NO_x) and sulfur oxides (SO_x) can increase allergic sensitization.[2,4]

Nitrogen oxides are generated by industrial combustions and oxidation processes or in the atmosphere by combined reactions with reactive oxygen species. In urban areas, its concentrations are usually high due to the automobile exhaust and heating processes. Sulfur dioxide is released into the atmosphere by natural (volcanic) or anthropogenic (coal burning, vehicle emissions) sources and, thus, can be classified as a point-source pollutant.[6] SO_2 is soluble in water, and once in contact with airborne oxygen, it changes to sulfuric acid. Exposure to NO_2 and other gases have been reported to interfere with plant metabolism, pollen viability, germination, tube elongation, anther development, and plant growth in some plant species.[7−9]

The most common pollinosis in Europe are caused by pollen from grasses, trees (birch, olive and cypress), and weeds.[10] Sensitization to pollen allergens is variable between different regions of the world, according to climate, urbanization levels, geography, and vegetation.[10] In the urban area of Porto, the

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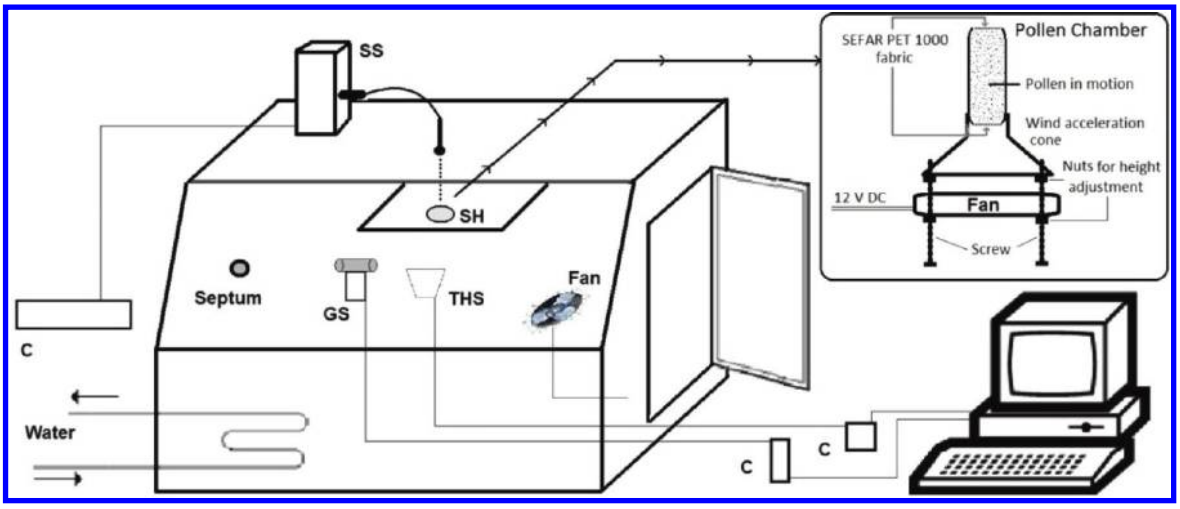

Figure 1. Schematic representation of the environmental chamber used for the exposure of *Acer negundo* pollen to SO_2 or NO_2. C, controller units; GS, gas sensor; SH, sample holder; SS, solar simulator; THS, temperature and humidity sensor.

Table 1. Results of Total Soluble Protein Content and Pollen Germination Rate of *Acer negundo* Pollen Exposed to SO_2 and NO_2[a]

treatment	control	SO_2				NO_2			
		Dy		St		Dy		St	
gas level	c	+	++	+	++	+	++	+	++
gas concentration (ppm)	–	0.12 ± 0.10	0.31 ± 0.24	0.11 ± 0.07	0.26 ± 0.21	0.10 ± 0.02	0.30[b]	0.15 ± 0.04	0.30[b]
protein concentration (μg/mL mg DW)	102.4 ± 0.3	99.7 ± 2.0	68.8 ± 8.3	77.6 ± 7.2	93.9 ± 3.3	94.8 ± 1.6	105.5 ± 2.2	107.4 ± 2.1	103.4 ± 1.6
germination (%)	58.2 ± 5.3	45.4 ± 5.0	45.2 ± 3.6	43.0 ± 5.5	41.6 ± 4.4	36.0 ± 5.3	36.2 ± 4.9	44.8 ± 7.7	35.6 ± 5.1

[a]Dy, dynamic experiment; St, static experiment. Gas level: c, control experiment; +, hour-limit value; ++, two times hour-limit value. DW, dry weight. [b]Values presented were estimated as some of them were above the sensor detection limit.

most common trees producing allergenic pollen are *Platanus* spp, *Populus* spp, *Quercus* spp, *Salix* spp., and *Acer negundo*.[11]

Acer negundo or maple ash (Aceraceae family) has been spread out for ornamental purposes as it is easy-growing and has been recorded in some aerobiological studies,[11,12] even though it has not received too much consideration regarding pollen allergenicity. Until now, no allergens have been characterized for this tree species. The aim of this research was to study the effects of SO_2 and NO_2 on protein content, allergenicity, and germination rates of in vitro exposed *Acer negundo* pollen. In order to that, we assembled a novel environmental chamber system and subjected pollen samples to dynamic or static situations at two different gas concentration values: just below the safety standard limits for human health protection and two times those levels.

MATERIAL AND METHODS

Pollen Samples. *Acer negundo* is present in public gardens and sidewalks scattered around Porto, which is the second largest Portuguese city. Located in the north, with approximately 263 thousand inhabitants, it is limited on the west by the Atlantic Ocean and on the south by the Douro River. The Porto region has a high industrial density, making this the highest power consumption per capita and per industry in the north of Portugal. The most important stationary sources of atmospheric pollutants are the oil, petrochemical, thermoelectric industries, as well as incineration units and shipping port activity. Also, pollutants emissions are recorded because of high motor traffic.

The anthers were collected during *Acer* flowering season, and after removal of other plant parts, they were dried at 27°C, gently crushed, and the pollen thus released was passed through different grades of sieves to obtain pure pollen. Pollen samples were then stored at −20 °C.

Patient Sera. Eighteen random atopic patients previously selected as sensitized to *Acer negundo*, assessed by skin-prick tests using this taxon pollen protein extracts, were chosen. Sera were separated from the whole blood, collected by venipuncture, and allergen specific IgE levels were measured by ImmunoCAP FEIA test (Phadia AB). The sera were chosen having specific IgE values between low and very high (<0.35−48.10 Ku/L). One serum from a nonsensitized patient to *Acer negundo* pollen was also used as a negative control.

Environmental Chamber and Pollen Exposure. Pollen samples were in vitro exposed to gases in an environmental chamber made of an acrylic and wood mix, 50 cm × 70 cm × 50 cm (Figure 1).

In this chamber, sunlight was simulated by a solar simulator (Newport Oriel 96000 150 W) with air mass filters 1.5 Global 81094 and a liquid light guide positioned 15 cm above the sample holder. This support was assembled 20 cm above the roof and centered within the chamber. It consists of a rectangle glass held by two metal frames screwed to the chamber wall

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that act as the base support for pollen samples. Five glass condensers were connected in serial so that the cool water circulating from an outside refrigerated water bath would prevent greater temperature fluctuations inside the chamber. A fan (SUNON SF23080AF) was used to homogenize the air inside the chamber. Air temperature and relative humidity were continually monitored using an EBRO EBI20 sensor. The gas concentrations were monitored throughout each assay by AEROQUAL Series 500 sensors, registering data every minute. These sensors were connected to an external computer.

In order to assess the effect of the exposition model of pollen to pollutants, two types of assays were conducted: static (St) and dynamic (Dy) (Table 1). In the static experiments, pollen was scattered on a Petri dish, preventing its overcrowding, and placed on the sample holder. This assay mimics what may occur on dehiscent anthers, with only part of the pollen surface exposed to pollutants. In the dynamic experiments, pollen grains were 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 (see zoom inset, Figure 1). This apparatus intends to mimic what happens in the atmosphere, when pollen is airborne.

Each pollen sample (120 mg of dry weight, DW) was exposed during 6 h. Pollen was exposed to two pollutants (SO_2 or NO_2), under two different concentrations. Gas concentrations were chosen on the basis of the European Union Directive 2008/50/EC of 21 May 2008 on ambient air quality and cleaner air for Europe. This directive indicates that the atmospheric hour-limit values acceptable for human health protection are 0.133 ppm for SO_2 and 0.106 ppm for NO_2. Pollen samples were individually exposed to approximately those concentration values and two times those values. SO_2 and NO_2 concentrations were attained by injecting small volumes of gas, obtained from bottles of compressed gas (Sigma-Aldrich) through septa placed at the front chamber wall using a micro syringe. Three injections of gas per assay were performed with a 2 h interval between them in order to prevent greater concentration fluctuations. Average concentration values and standard deviation are presented (Table 1). A nonexposed pollen sample was used as control.

Protein Extraction and Quantification. Pollen grains (50 mg DW) were suspended in 1:20 (w/v) phosphate buffer saline at pH 7.4 at 4 °C. Total soluble proteins were extracted in the same buffer by continuous stirring for 4 h. The suspension was then centrifuged at 13200 rpm for 30 min at 4 °C. The supernatant was filtered through a 0.45 μm Millipore filter and centrifuged once again. The soluble protein content of all pollen extracts was quantified colorimetrically with the Coomassie Protein Assay Reagent (Pierce) by the Bradford method.[13] Three replicate measures were performed and the average and standard deviation values presented (Table 1).

SDS-PAGE and Immunoblots. Proteins from pollen extracts were separated in 12.5% polyacrylamide gels under reducing conditions[14], and the proteins were visualized by Coomassie Brilliant Blue R-250 staining. The molecular weight of protein bands was estimated by comparison with an established protein marker (PageRuler Plus Prestained Protein Ladder, Fermentas). For immunoblotting analysis, the protein was electroblotted onto nitrocellulose membranes (Protran, Whatman Schleicher and Schuell, Germany). The membranes were saturated during 1 h in a blocking solution (5% nonfat dry milk (w/v), 0.1% goat serum (v/v), in 20 mM Tris, 150 mM NaCl (TBS) and 0.1% Tween) and then incubated overnight at 4 °C with sensitized and nonsensitized patient sera to *Acer negundo* pollen diluted 1:10 in blocking solution for the identification of allergens. After washing, bound specific IgE were detected by horseradish peroxidase-conjugated antihuman IgE serum (Southern Biotechnology Associates). An ECL solution (Luminata Crescendo, Interface, Lda.) was used as a detection system. The chemiluminescent signal was exposed to AGFA medical X-ray film and developed by Fuji medical film processor model FPM 100A. The antigenic profile bands of the SDS-PAGE and immunoblots were quantified by a Molecular Imager GS800 calibrated densitometer and Quantity One 1-D Analysis, v4.6 (Bio-Rad Laboratories).

Pollen Germination Rate. Nonexposed and exposed pollen samples were germinated at 25 °C in the dark for 48 h in an autoclaved germination medium optimized by the authors (100 ppm H_3BO_3, 100 ppm $CaCl_2$, 15% sucrose, 0.5% agar, and adjusted pH 5.0). Two replicates were performed, and in each one, five random fields were counted (100 pollen grains per field) using a light microscope (Leica DMLB). A pollen grain was classified as germinated when the tube was longer than the pollen size.[15]

RESULTS AND DISCUSSION

Air pollution can interfere with the respiratory system directly by inducing acute effects on subjects with respiratory allergies.[3] However, its indirect effects on airborne allergens such as pollen proteins are still under study.

In the present study, different values in total soluble protein content were observed between nonexposed and exposed samples of *Acer negundo* pollen extracts (Table 1).

The SO_2-exposed pollen samples showed lower protein content compared with the control sample. However, the diverse values observed either among SO_2 concentration levels or exposure conditions (dynamic vs static) reveal an inconsistent effect, and it is difficult to ascertain, in this case, the influence of this pollutant in protein content. Regarding NO_2-exposed pollen samples, except for the Dy+, it was observed slightly higher protein content compared with the control sample, regardless of the exposure conditions. In the literature, both an increase and decrease in the total soluble protein content of pollen exposed to pollutants was reported. Some authors stated an initial increase in pollen protein content in *Argemone mexicana*[16] and *Ricinus communis*[17] after artificial exposure to SO_2 compared to nonexposed pollen. Besides, these authors also verified a subsequent decrease in protein content values with escalating concentration levels and time exposures to SO_2 and NO_2. Other studies also demonstrated a decrease in soluble protein contents of *Lagerstroemia indica*,[18] *Canna indica*,[19] *Phleum pratense*,[20] and *Thuja orientalis*[21] pollen from polluted areas against non-polluted ones.

In the present results, despite the differences in protein content observed, the electrophoretic profile revealed several bands ranging from 100 to 13 kDa after Coomassie staining of the gel with similar results between nonexposed and exposed pollen samples (Figure 2). Some studies also reported no changes in the electrophoretic profile between nonpolluted and polluted pollen.[21] However, Majd et al.[19] reported the disappearance of protein bands around 22 and 45 kDa from the SDS-PAGE profile of *Canna indica* pollen exposed to a polluted area in Tehran. Other authors observed a reduction in staining intensity and disappearance of protein bands from

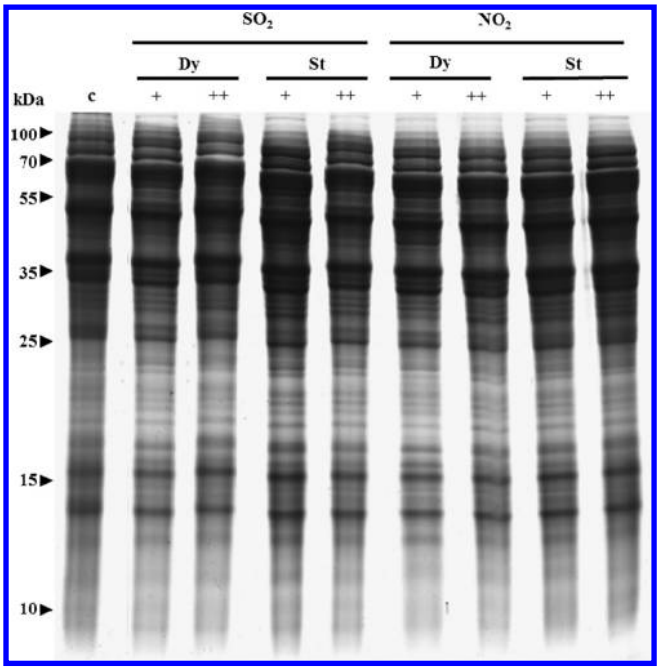

Figure 2. SDS-PAGE of total soluble proteins of *Acer negundo* pollen extracts exposed to SO_2 or NO_2. Dy and St, dynamic and static experiments, respectively. Signs above the lanes represent gas concentration level: c, control experiment; +, hour-limit value; ++, two times hour-limit value.

pollutant exposed pollen in comparison to nonpolluted one.[16,18] Taken together, these studies suggest that protein content and profile modifications after pollutant exposure may be dependent on species and/or gas type and concentrations that may lead to contradictory outcomes.

To verify the effects of SO_2 and NO_2 on pollen allergenicity, 18 immunoblots were assayed using different patient sera sensitized to *Acer negundo* pollen (only three are shown in Figure 3, representing the situations of lowest, average, and highest reactivity).

Pollen allergenic changes were evaluated according to the binding affinity to specific IgE of atopic patient sera to pollen extracts. Higher binding affinities indicate higher IgE reactivity, revealing more conspicuous bands with higher optic densities (Figure 4). Comparing these results, it is possible to ascertain the influence of SO_2 and NO_2 on pollen allergenicity. Even though no clear differences were observed between nonexposed and exposed samples in the SDS-PAGE, the immunoblots presented evident differences. Pollen exposure to SO_2 or NO_2, regardless of the exposure conditions, lead to higher IgE reactivity and, therefore, potential higher allergenicity compared with nonexposed pollen. Moreover, in the exposed pollen samples, it was observed the presence of bands that were absent in the nonexposed pollen, indicating that some proteins only began to be reactive after the exposure to the pollutant. The common bands recognized in nonexposed and exposed pollen were always less intense in the former (Figures 3 and 4).

Air pollution can be regarded as a stress factor and, under stress conditions, the plants may up-regulate the expression of certain proteins such as pathogenesis-related proteins, an integral part of plant defense system, as was reported for *Cupressus arizonica* pollen collected from a high traffic area in Toledo, Spain.[22] Besides, air pollutants like NO_2 can modulate the expression of some pollen allergens by post-translational modifications.[23] An in vitro study performed on *Phleum pratense* pollen exposed it to concentrations of SO_2 and NO_2 higher than the levels used in our assay (10-fold), revealing a reduction of released allergens and a significant reduction of IgE reactivity after pollutant exposure. By this, these authors stated that allergen decrease could be induced by a mechanical loss of grass allergens or altered biochemical properties of pollen grains, decreasing IgE recognition of patient sera.[20] Our results showed different IgE reactivity behavior depending on the experiments performed. Considering the pollen exposure to SO_2, a trend was observed with higher reactivity being obtained when pollen was exposed in the dynamic experiment to higher pollutant concentrations (0.31 ppm). Similarly, more than 50% of the tested sera revealed higher IgE recognition at both pollutant concentrations tests in the dynamic experiment comparing with the static one.

A higher reactivity in NO_2-exposed pollen in the dynamic experiment at higher pollutant concentrations (0.30 ppm) was observed for all sera tested. These results were very similar to the ones obtained for both concentrations (0.30 and 0.15 ppm) in the static experiment. In fact, airborne NO_3 radicals formed upon reaction of NO_2 with O_3 can nitrate the proteins contained in biogenic aerosol particles, such as pollen, enhancing allergenicity in polluted urban environments and may also serve as an environmental marker to trigger the immune system.[23,24] In this assay, the sample exposed to the lowest concentration in the dynamic experiment (Dy+) revealed visible smaller number and intensity of reactive bands in all tested sera, contrasting to the results of the other samples. We hypothesize that this particular sample may have been damaged during or between biochemical assessments because its protein content is very different from other samples exposed to NO_2. Unfortunately, it was not possible to repeat the assays because each serum represents only one patient with a regular volume of around 0.5−0.7 mL, which supplies one single experiment. However, it can be reasoned that an analogous immunoreactive profile could be expected for Dy+ given the similar results obtained for other samples.

Our results demonstrated that *Acer negundo* pollen contains multiple potentially allergen proteins that can be modified by the interaction with air pollutants. All sera, regardless of the amount of specific IgE for *Acer negundo* pollen, recognized five main sets of bands in exposed samples in accordance with SDS-PAGE results (Figure 3). Our results also showed that among the 18 patient sera used, 88.9% reacted to a protein band around 50 kDa, 100% presented a band of around 41 kDa, 88.9% reacted to a band around 32 kDa, 83.3% showed a band of around 19−17 kDa, and 33.3% of sera recognized 15 and 13 kDa protein bands. An immunological profile of *Acer negundo* pollen has already been reported,[11] but the identification and characterization of its allergens at the molecular level are still to be studied.

Until now, almost all the in vitro experiments conducted by other authors on pollen exposed to pollutants used higher concentrations than those regularly observed in the environment. In our study, the lower levels tested correspond to values below the target limit value for the human health protection and then give us the indication that, at these concentrations, modifications may occur in the pollen proteomics that will lead to an exacerbation of pollen allergies. Therefore, conditions combining the presence of allergenic airborne pollen with peaks of atmospheric concentrations of SO_2 or NO_2 may lead to an intensification of allergenic reactions in certain individuals.

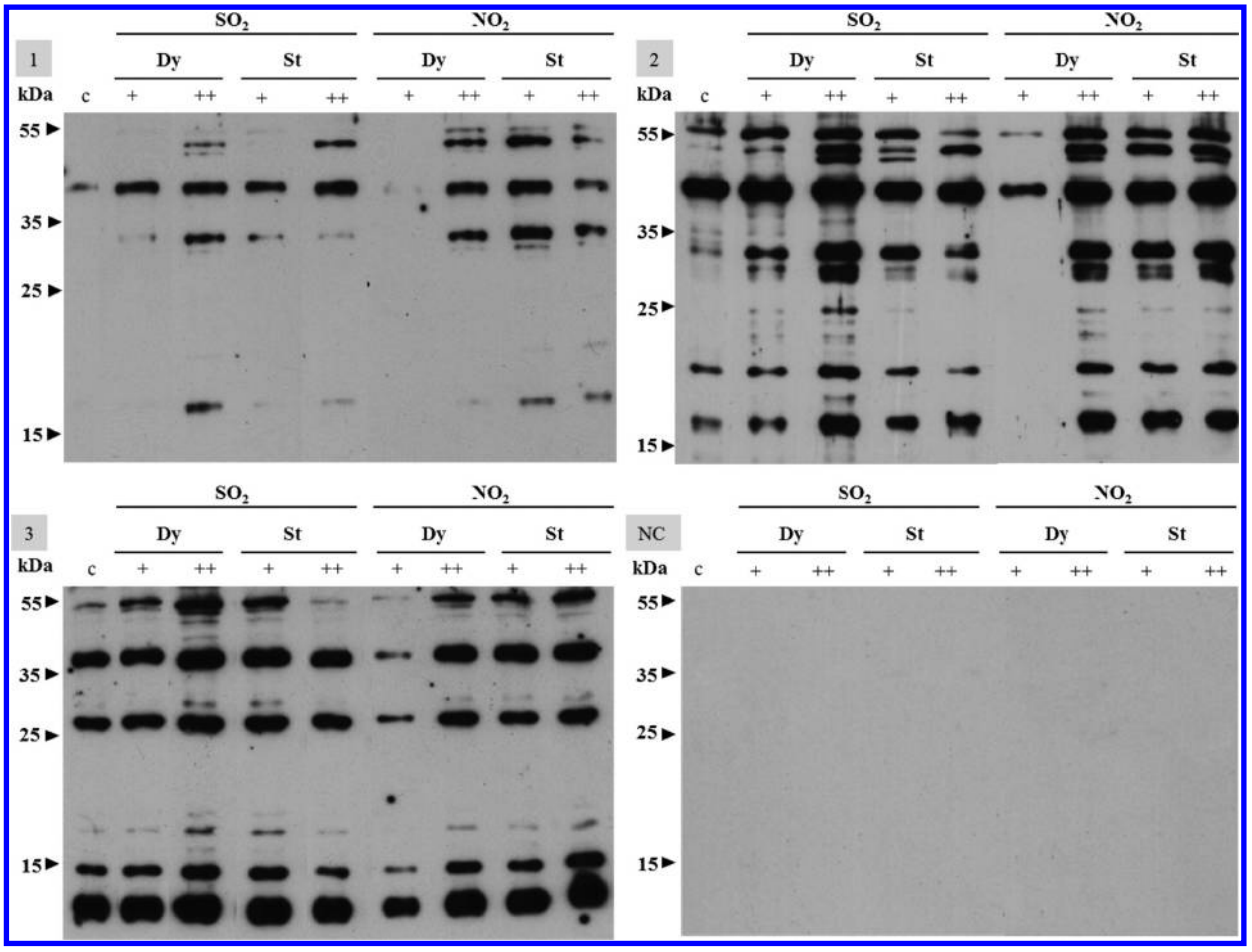

Figure 3. Immunoblots of *Acer negundo* pollen extracts exposed to SO_2 or NO_2. Dy and St, dynamic and static experiments, respectively. Signs above the lanes represent gas concentration level: c, control experiment; +, hour-limit value; ++, two times hour-limit value. Three patient sera (1, t1 0.21 Ku/L; 2, t1 9.13 Ku/L; 3, t1 48.1 Ku/L) and a negative control (NC) were used.

In Table 1 are shown the results of germination rates of *Acer negundo* pollen before and after exposure to pollutants. A significant statistical reduction ($p < 0.00$) was observed in the germination rate of pollen exposed to SO_2 and NO_2 compared to nonexposed pollen. After the SO_2-exposed, the pollen germination was inhibited in around 10%, while for NO_2-exposed a reduction of about 20% was observed. The germination rates of pollen exposed to SO_2 and NO_2 were significantly different ($p < 0.05$). For each gas (SO_2 and NO_2), no significant statistical effect ($p < 0.05$) was observed between the dynamic and static experiments, as well as between the different gas concentration levels tested.

Thus far, some experiments reporting a negative effect on pollen germination after exposure to SO_2 and NO_2 have been performed. For *Lepidium virginicum* L., it was reported an in vivo pollen germination reduction of 50% when pollen was exposed to 0.6 ppm of SO_2 for 2, 4, and 8 h during flowering.[8] Also, for *Lilium longiflorum* pollen, a marked inhibition of tube elongation was verified when exposed to 0.71 ppm SO_2 or 2 ppm NO_2.[9] Chichiriccó and Picozzi[7] exposed plants of *Crocus vernus* to 0.2−11 ppm NO_2 and observed a pronounced inhibition of pollen germination, but then after cessation of exposure, they verified a restoration of germinative capacity. The SO_2 and NO_2 concentrations tested in our study were much lower than the ones reported on the above-mentioned experiments. Hence, our results indicated that concentrations just below the target limit value for the protection of human health in Europe affected pollen germination and may play an important role in the plant reproduction process. These results also pointed out a higher influence of NO_2 on pollen germination than SO_2, despite the similar concentrations tested. NO_2 is chemically more reactive than SO_2 and can probably induce more effective modulation in protein expression and in protein behavior, which is what concerns physiological metabolism, such as pollen germination. Nonetheless, to ascertain if one gas would be more harmful than the other, an in vivo pollination test should be performed in order to confirm the behavior of pollen under natural conditions. If the proteins involved in pollen germination correspond to the antigenic ones, the specific modifications occurring still need to be identified.

In conclusion, *Acer negundo* pollen antigens are affected by SO_2 and NO_2 under in vitro conditions, supporting the idea of an interaction between pollen and air pollutants outside the organism, which in turn can promote pollinosis-related symptoms. These interactions occurred at concentrations around the standard limit values for the human health protection, indicating the importance of considering the synergistic effects between the constituents of the aerosol when drawing the limit protection values to protect the most vulnerable groups of the population, especially susceptible individuals to respiratory allergies. Regarding plant sexual reproduction, both pollutants affected pollen germination. The proteins associated with germination that are influenced by air

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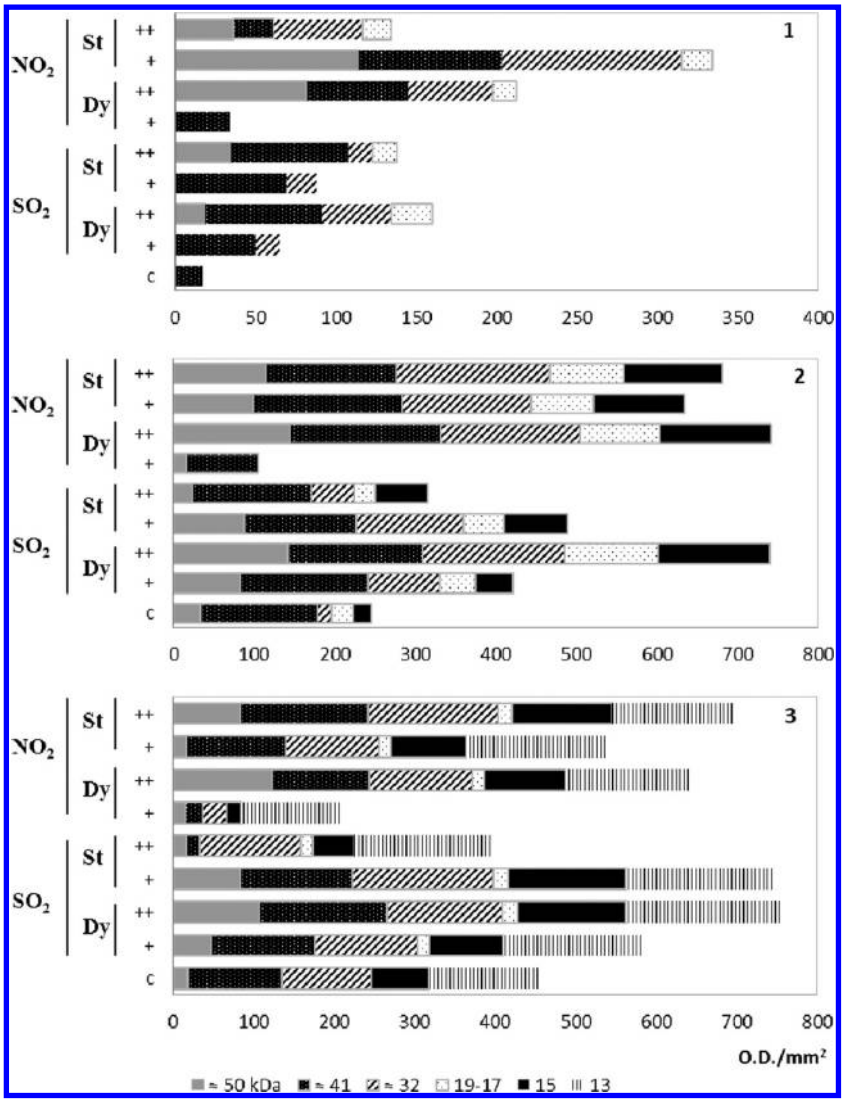

Figure 4. Optic densities (O.D.) of IgE-reactive bands of *Acer negundo* pollen protein extracts exposed to SO_2 or NO_2. Dy and St, dynamic and static experiments, respectively. Signs on the left represent gas concentration level: c, control experiment; +, hour-limit value; ++, two times hour-limit value. Three patient sera (1, t1 0.21 Ku/L; 2, t1 9.13 Ku/L; 3, t1 48.1 Ku/L) were used.

pollution and those responsible for allergic symptoms of *Acer negundo* pollen would enable an interesting subject for future study and the constant improvement of clinical knowledge.

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