

Effects of CO₂ on *Acer negundo* pollen fertility, protein content, allergenic properties, and carbohydrates

M. Silva · H. Ribeiro · I. Abreu · A. Cruz ·
J. C. G. Esteves da Silva

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Abstract Atmospheric gaseous pollutants can induce qualitative and quantitative changes in airborne pollen characteristics. In this work, it was investigated the effects of carbon dioxide (CO₂) on *Acer negundo* pollen fertility, protein content, allergenic properties, and carbohydrates. Pollen was collected directly from the anthers and in vitro exposed to three CO₂ levels (500, 1000, and 3000 ppm) for 6 and 24 h in an environmental chamber. Pollen fertility was determined using viability and germination assays, total soluble protein was determined with Coomassie Protein Assay Reagent, and the antigenic and allergenic properties were investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunological techniques using patients' sera. Also, pollen fructose, sucrose, and glucose values were

determined. Carbon dioxide exposure affected negatively pollen fertility, total soluble protein content, and fructose content. The patient sera revealed increased IgE reactivity to proteins of *A. negundo* pollen exposed to increasing levels of the pollutant. No changes were detected in the SDS-PAGE protein profiles and in sucrose and glucose levels. Our results indicate that increase in atmospheric CO₂ concentrations can have a negative influence of some features of *A. negundo* airborne pollen that can influence the reproductive processes as well as respiratory pollen allergies in the future.

Keywords Atmospheric pollution · Respiratory allergies · Pollen germination · Pollen viability · IgE · Sugars

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M. Silva · J. C. G. Esteves da Silva
Department of Chemistry and Biochemistry, Faculty of Sciences,
University of Porto, Porto, Portugal

H. Ribeiro (✉) · I. Abreu
Geology Centre of University of Porto, R. Campo Alegre 687,
4169-007 Porto, Portugal
e-mail: helenaribeiro@fc.up.pt

I. Abreu
Biology Department, Faculty of Sciences, University of Porto, Porto,
Portugal

A. Cruz
Clinical Pathology Service, Immunology Laboratory, Vila Nova de
Gaia Hospitalar Centre, Vila Nova de Gaia, Portugal

J. C. G. Esteves da Silva
Centre of Investigation in Chemistry (CIQ-UP), University of Porto,
Porto, Portugal

Introduction

Pollen grains are produced during the reproductive cycle of the higher plants to perform the vital task of sexual reproduction. In anemophilous plants, these biological structures are released into the atmosphere in consequence of the pollination process during flowering season. While airborne, pollen is subjected to chemical and physical interactions with other atmospheric constituents that can stress both the pollinating plant and the pollen grain (Sofiev et al. 2013). In the past years, the interaction between airborne pollen and gaseous pollutants such as O₃, SO₂, NO₂, or CO has been studied in order to access the qualitative and quantitative changes in pollen features (e.g., Cuinica et al. 2014; Ribeiro et al. 2014; Sousa et al. 2012). Pollen grains can enter people's airways and induce respiratory allergic reactions on susceptible individuals due to the presence of allergens not only in the inner part of the pollen (intine and cytoplasm) but also in the pollen outer wall (submicroscopical sites of the exine and orbicules) (Linskens and Cresti 2000). The increase in respiratory allergies has been usually related with environmental exposure and

includes many diverse causes related with air quality (Ring 2012). Also, environmental changes resulting from the increased concentrations of greenhouse gases have received significant attention in recent times due to its impact on the biotic systems, including both direct and indirect effects on human health (Rogers et al. 2006). One of the most abundant greenhouse gases in the atmosphere (excluding water vapor) is carbon dioxide (CO₂), with concentrations increasing since the Industrial Revolution, being around 30 % higher than the preindustrial values at around 390 ppm (Dlugokencky and Tans 2014). IPCC projections are for CO₂ concentrations to continue rising to as much as 500–1000 ppm by the year 2100 (IPCC 2007; Taub 2010).

CO₂ can be naturally produced as part of the Earth's carbon cycle (the natural circulation of carbon among the atmosphere, oceans, soil, plants, and animals) or be a product of anthropogenic sources such as fossil fuel combustion, limestone burning, and deforestation (Jackson 2003). Human-related CO₂ emissions impact on the carbon cycle—both by adding more CO₂ to the atmosphere and by influencing the ability of natural sinks to remove CO₂—are pointed to be mainly responsible for the increase occurred in the atmosphere since the industrial revolution (NRC 2010).

Plants response to atmospheric CO₂ concentration depends strongly on the timescale over which the response is measured. Plant leaf-level photosynthesis, stomatal conductance, and respiration rates can directly and immediately respond to changes in atmospheric CO₂ concentration (Medlyn and McMurtrie 2005). These physiological changes have impact on plant growth, phenology, and productivity. Some experiments exposing plants to an elevated CO₂ atmosphere reported an anticipation of the flowering season with not only the increase production of pollen and the duration of the pollination period but also a decrease in pollen fertility (Blando et al. 2012). These changes in onset, duration, and intensity of the pollen season will have an immediate influence on the quality of life of allergic individuals (Dahl et al. 2013). However, to our knowledge, the effects of CO₂ on pollen while airborne were not yet investigated. Therefore, the aim of this research was to study the effects of three distinct concentration levels of CO₂ on protein content, fertility rates, IgE-binding protein profiles, and soluble carbohydrate content of in vitro-exposed *Acer negundo* pollen. This plant species was selected because it has been spread out for ornamental purposes and its pollen is considered allergenic.

Material and methods

Pollen samples

Pollen from *A. negundo* was collected during the flowering period in public gardens or sidewalks of the second largest

Portuguese city—Porto. The trees selected were easy to access, free standing, and presented flowers with dehiscent anthers. In each tree, anthers were randomly collected from all quadrants of the tree in different branches. In the laboratory, the anthers were dried at 24 °C and gently crushed, and the pollen thus was released passing through different grades of sieves to obtain a pure pollen. The collected pollen was stored together at −20 °C.

In vitro pollen exposure to CO₂

An environmental chamber system (50×70×50 cm), previously described in Sousa et al. (2012), was used to expose pollen samples to carbon dioxide (CO₂). 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). The reproducibility of exposure conditions was demonstrated in Ribeiro et al. (2013).

For each exposition experiment, a pollen sample of 250 mg of dry weight—DW—was placed in a Falcon tube with the bottom cut and 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. This sampling system allows the circulation of the air through it and the resuspension of the pollen.

Pollen samples were individually exposed to CO₂ at approximately equal (500 ppm), double (1000 ppm), and six times (3000 ppm) the average atmospheric concentration value (Table 1) observed in Porto city. Each experiment was conducted during two different time periods, for 6 and 24 h. CO₂ concentrations were obtained by chemical reaction of chloride acid with sodium bicarbonate that were mixed in an Erlenmeyer, at stoichiometric amounts. The formed gas was subsequently injected into the chamber. The injections of gas per assay were performed using a microsyringe within each 2-h interval in order to prevent greater concentration fluctuations. Gas concentrations were continuously monitored at every minute using a CO₂ gas concentration sensor (AEROQUAL Series 500 sensors, 0–5000 ppm).

Table 1 CO₂ levels, temperature, and relative humidity registered during the experiments of *Acer negundo* pollen in vitro exposure

CO ₂ level (ppm)	Time (h)	Gas concentration (ppm)	T (°C)	RH (%)
500	6	596±40	27.4±0.5	43.2±2.1
	24	515±24	26.3±0.7	44.4±1.9
1000	6	1066±141	25.5±0.9	44.4±0.3
	24	1028±270	26.8±0.9	46.0±0.5
3000	6	2987±279	27.2±1.2	45.7±0.7
	24	2502±926	26.0±1.0	42.2±0.2

Pollen fertility

Pollen fertility was determined using viability and germination assays.

For each experiment, pollen viability was estimated using the trypan blue dye (TBD—Sigma®), which is a vital dye that interacts with cells only if damage of the membrane occurs and therefore stains blue nonviable pollen samples. Pollen grains (2 mg) were placed for 5 min in 0.5 ml of 60 % trypan blue and centrifugation for 3 min at 16,300g. Then, 20 µl were placed on a microscope slide and observed at light microscope (DMLB: Leica). Pollen viability was determined as the percentage of noncolored pollen relative to the total pollen. For each sample, one slide was prepared, and for each slide, three random fields (100 pollen grains per field) were counted.

Pollen was in vitro germinated in an optimized germination media (100 ppm H₃BO₃, 100 ppm CaCl₂, 15 % sucrose, and 0.5 % agar at pH 5.0) incubated in the dark at 27 °C for 48 h.

After incubation, three random fields were counted (100 pollen grains per field) using a light microscope (Leica DMLB). A pollen grain was classified as germinated when its tube was longer than the pollen's diameter (Nepi and Franchi 2000).

Protein extraction and estimation

Pollen extracts were prepared by incubating in 1:20 (w/v) phosphate buffer saline at pH 7.4 with continuous stirring for 4 h at 4 °C. The suspension was then centrifuged at 16,100g 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 estimated colorimetrically with Coomassie Protein Assay Reagent (Pierce) by Bradford's method (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 and standard deviation values presented (Table 2).

SDS-PAGE and immunoblots

Proteins from pollen extracts were separated in 12.5 % (w/v) polyacrylamide gels under reducing conditions (20 µg/ml per lane), and the proteins were visualized by Coomassie Brilliant Blue R-250 staining. The molecular weight of protein bands was estimated by comparison with an established protein marker (PageRuler™ Plus Prestained Protein Ladder, Fermentas). For immunoblotting analysis, the protein bands separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 30 µg/ml per lane) were electroblotted (TE22 Mighty small transphor unit—GE Healthcare) to nitrocellulose membranes (Protran, Whatman® Schleicher & Schuell, Germany). Transfers were carried out in a solution of 192 mM glycine, 25 mM Tris, and 20 % methanol (transfer buffer) during 2.5 h at 200 mA. The membranes were saturated during 1 h in a blocking solution (5 % nonfat dry milk and 0.1 % goat serum in TBS-T (20 mM Tris, 150 mM NaCl with 0.1 % Tween-20)) and then incubated overnight at 4 °C with atopic patient sera diluted 1:10 in blocking solution for the identification of allergens. After washing, bound specific IgE were detected by alkaline phosphatase-conjugated mouse antihuman IgE serum (1:2000) (Southern Biotechnology Associates) during 1 h at

Table 2 Average and standard deviation in fertility (germination and viability) and total soluble protein content of *Acer negundo* pollen exposed to different levels of CO₂ during two time periods (6 and 24 h). *t* test (*p* values) for differences in the experimental parameter

	Time (h)	CO ₂ level (ppm)			ANOVA (<i>p</i> value)
		500	1000	3000	
Germination (%)	6	45.3±3.1a	36.7±3.8b	28.7±2.5c	0.02
	24	36.7±1.5a	28.0±4.0b	18.7±1.5c	0.00
<i>t</i> test (<i>p</i> value)		0.01	0.05	0.00	
Viability (%)	6	53.3±4.9a	38.3±1.5b	29.0±2.6c	0.00
	24	38.3±1.5a	32.3±2.1b	22.0±2.0c	0.00
<i>t</i> test (<i>p</i> value)		0.00	0.02	0.02	
Protein (µg/ml)	6	4679.3±567.8a	4884.8±88.5a	6161.8±222.8b	0.04
	24	5462.1±211.9a	5745.9±370.5a	4850.6±293.2b	0.03
<i>t</i> test (<i>p</i> value)		0.09	0.02	0.00	

Values lower than 0.05 indicate the rejection of the null hypothesis at the 95 % confidence level. Different letters indicate statistically significant differences given by the ANOVA test followed by the LSD post hoc test

values between each exposure time. ANOVA *p* values for differences in the experimental parameter values between CO₂ levels during the same exposure time

4 °C. The reaction was revealed using Novex® AP chromogenic substrate (Invitrogen).

The antigenic profile of the SDS-PAGE and immunoblots were quantified using the software myImageAnalysis™ (© Thermo Fisher Scientific Inc.).

Patient sera

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

Carbohydrates

The soluble carbohydrate content was quantified in the pollen samples individually exposed for 6 and 24 h to CO₂ at approximately equal (500 ppm) and double (1000 ppm) the average atmospheric concentration value observed in Porto city.

Samples were suspended in 1:20 (*w/v*) phosphate buffer saline at pH 7.4 with continuous stirring for 4 hours at 4 °C. The suspension was then centrifuged at 16,100*g* for 30 min at 4 °C. The supernatant was filtered through a 0.45-μm Millipore filter and centrifuged once again. The soluble carbohydrate fructose, sucrose, and glucose values were determined using a Sigma-Aldrich kit FA20, SCA20, and GAHA20, respectively. Each kit only allowed performing four single experiments. In order to ensure the determination of the carbohydrate content in similar experimental conditions, it was decided to test only the two lower CO₂ concentrations.

Statistical analysis

Values are presented as means±standard deviation of *n* experiments. The normality of the data was evaluated by Shapiro-Wilk test.

To evaluate the quantitative effects of increasing CO₂ levels during the same exposure time on the experimental parameters, a one-way ANOVA was used. When significant differences were detected, the LSD post hoc test was selected to perform pairwise multiple comparisons of three averages.

To evaluate the quantitative effects of different exposure times to the same CO₂ level on the experimental parameters, a Student's *t* test was used.

The SPPSTM Statistic program (© IBM Inc.) was used to perform the analysis, and values of *p*<0.05 were considered significant.

Results

Differences were observed in the fertility and total soluble protein content of pollen of *A. negundo* after exposure to CO₂ (Table 2). Pollen viability and germination rates were affected with exposure to CO₂. A significant reduction in these pollen features was observed not only between increasing concentrations of CO₂ but also between the two tested exposure times regardless of the gas concentration used.

In the pollen total soluble protein content, no significant differences were observed between pollen exposed to 500 and 1000 ppm of CO₂. Only values of 3000 ppm caused significant changes in protein content. However, these changes were different depending on the exposure time. When pollen was exposed to the highest tested CO₂ level for 6 h, an increase in protein content was observed, while pollen exposed for 24 h presented an opposite behavior (Table 2).

SDS-PAGE results reveal a similar polypeptide profile between the exposed pollen samples of *A. negundo* to different levels of CO₂ (Fig. 1). The electrophoretic profile showed multiple bands of molecular weights ranging from 140 to 14 kDa.

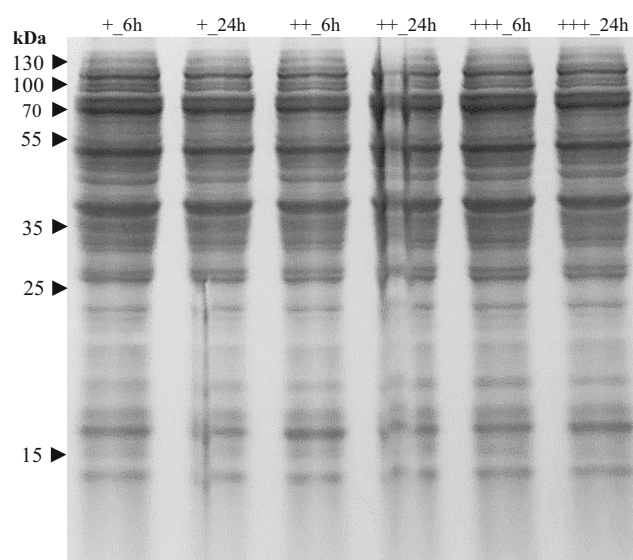


Fig. 1 SDS-PAGE of total soluble proteins of *Acer negundo* pollen extracts exposed to CO₂. Signs above the lanes represent gas concentration level: + 500 ppm, ++ 1000 ppm, +++ 3000 ppm, and time of exposure (6 and 24 h). Molecular weights of bands expressed in kilodalton

Through 1-DE immunoblotting, 14 IgE-reactivity bands in *A. negundo* were observed (Fig. 2). The most common reactive IgE protein bands were the ones around 51–59, 42–49, and 30–35 kDa. Other allergenic polypeptides in the range of 70 and 12–15 kDa were also detected.

An increase in IgE-binding pattern of all the atopic patients' sera with increasing time and concentration of CO₂ was observed.

Finally, a decrease in the pollen fructose content with increasing CO₂ concentration and exposure time was observed. In fact, the decrease was significant between pollen exposed to 500 ppm of CO₂ and 1000 ppm for 6 h (Table 3). For glucose, an increase in its levels was observed when both the time of exposure and CO₂ concentration were augmented,

although with no statistical significance. Also, sucrose levels did not vary significantly but in this case, the variation pattern was not consistent as observed for the other two determined carbohydrates.

Discussion

Pollutant gases such as O₃, SO₂, and NO₂ have been pointed out to affect several pollen features. Many studies have reported a decrease in pollen viability, germination, and total soluble protein content in several plant species after pollen exposure to either in vivo conditions of high pollution levels or in vitro exposure to variable concentrations of a specific

Fig. 2 Optic densities (intensity/area) of most prominent IgE-reactive bands observed after immunodetection of *Acer negundo* pollen extracts exposed to distinct CO₂ concentration levels and times assayed with a panel of six sera from different sensitized individuals. Signs in the legend represent gas concentration level: + 500 ppm, ++ 1000 ppm, +++ 3000 ppm, and time of exposure (6 and 24 h)

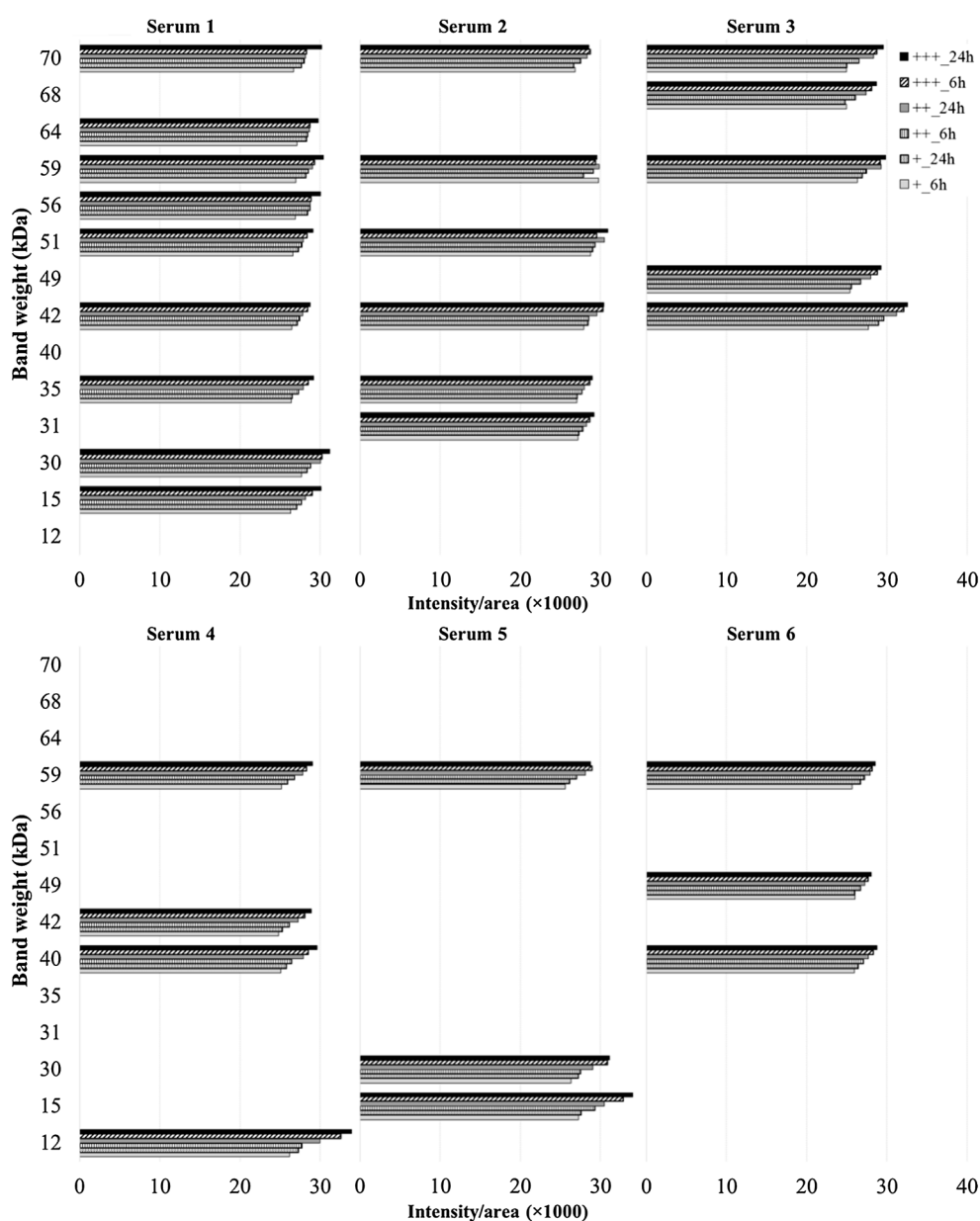


Table 3 Average and standard deviation in soluble carbohydrate content (fructose, sucrose, and glucose) of *Acer negundo* pollen exposed to two different levels of CO₂ during two time periods (6 and 24 h). *t* test (*p* values) for differences in the experimental parameter values between each exposure time and between CO₂ levels during the same exposure time

Carbohydrates (μg sugar/mg pollen)				
	Time (h)	CO ₂ level (ppm)		<i>t</i> test (<i>p</i> value)
		500	1000	
Fructose	6	72.5±1.0	64.0±0.6	0.03
	24	66.8±8.8	58.1±3.3	0.50
<i>t</i> test (<i>p</i> value)		0.59	0.22	
Sucrose	6	67.8±3.4	59.0±5.8	0.35
	24	62.7±1.7	63.6±4.1	0.86
<i>t</i> test (<i>p</i> value)		0.31	0.58	
Glucose	6	54.1±2.3	56.2±0.1	0.64
	24	56.5±1.2	57.6±0.6	0.54
<i>t</i> test (<i>p</i> value)		0.57	0.15	

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

gaseous pollutant such as O₃ or NO₂ (e.g., Cuinica et al. 2014; Rezanejad 2009; Sousa et al. 2012). However, experiments on pollen with CO₂ are to our knowledge inexistent.

Atmospheric CO₂ increase has been pointed as responsible for changes in several aspects of plant physiology and development (Taub 2010; Ziska and Beggs 2012).

The influence of CO₂ on pollen fertility has been mainly tested by growing plants under an increasing CO₂ atmosphere and comparing its pollen viability or germination with those plants grown under current ambient conditions (Taub 2010). Results showed a decrease in pollen germination, deficient pollen tube elongation, or even complete sterility (Blando et al. 2012). In our experiments, a negative relation between viability and germination rates with increasing CO₂ concentrations and exposure times was observed, showing a significant direct effect of this gas on pollen.

The negative influence on several species pollen germination when in vitro exposed to SO₂, NO₂, CO, and O₃ has been a common result of studies conducted on this subject (Chichiricco and Picozzi 2007; Cuinica et al. 2014; Dubay and Murdy 1983; Sousa et al. 2012). Our results could indicate that also CO₂ can contribute to this pollen fertility reduction.

Free-air carbon dioxide enrichment (FACE) experiments showed a decrease in plant's tissue concentrations of nitrogen and protein under elevated CO₂ (Ainsworth and Long 2005). Therefore, it can be expected that pollen protein content could be affected in the same manner when exposed to high concentrations of CO₂. In our study, the pollen exposed to levels of 500 and 1000 ppm presented similar protein content;

however, when exposed to 3000 ppm for 24 h, the total soluble protein content significantly decreased. These results can indicate that a CO₂ enriched atmosphere may have also an influence on pollen protein content consistent with the one observed in the FACE experiments.

A. negundo pollen exposed for 6 h to 3000 ppm of CO₂ presented an initial increase in protein content contrary to the decrease observed when exposed for 24 h. This behavior has also been reported in *Argemone mexicana* (Parui et al. 1998) and *Ricinus communis* (Bist et al. 2004) with escalating time exposures to SO₂ and NO₂ and might be related with a stress response of the pollen.

Furthermore, the total soluble protein content decrease observed in our study is in accordance with the idea that atmospheric pollutant gases, including CO₂, affect pollen protein content negatively. However, the degree of this influence can be dependent, among other factors, on the gas concentration (Ribeiro et al. 2014).

Despite the differences observed in the pollen-soluble protein content, no changes were observed in their electrophoretic profile between the different experiments.

Different results have been reported in the literature concerning the comparison of the pollen protein electrophoretic profile between non-polluted and polluted pollen. It has been described from no changes to the disappearance of protein bands or even reduction in staining intensity (Majd et al. 2004; Rezanejad 2009; Ribeiro et al. 2014). This suggests a dependency of factors such as the plant species, gas type, or concentration on the protein profile modifications, which it is likely to happen also in the case of CO₂.

The exposure of pollen to pollutants has been suggested to alter pollen biochemical characteristics, increasing its allergenic potential, thus leading to an aggravation of allergic symptoms in susceptible individuals or the development of symptoms in non-symptomatic ones (Bosch-Cano et al. 2011; Rezanejad 2009; Sousa et al. 2012).

Even though no electrophoretic profile changes were observed in our experiments, an increase in IgE reactivity of patient sera to pollen protein extracts was observed with the increasing time and concentration of CO₂, which in turn could be related with increasing allergenicity. However, these reactivity changes were smaller when compared with changes obtained for *A. negundo* pollen when exposed to high levels of SO₂, NO₂, or O₃ (Ribeiro et al. 2014; Sousa et al. 2012). This could suggest that CO₂ may have a lower impact on pollen allergenicity when compared with the influence of other atmospheric pollutant gases. Nonetheless, in pollen of *Artemisia artemisiifolia* grown at increasing atmospheric CO₂ concentrations, a significant increase in Amb a 1 allergen was observed although total pollen protein remained unchanged (Singer et al. 2005). These results may suggest that recent and projected increases in atmospheric CO₂ concentrations could directly contribute to an exacerbation of respiratory pollen

allergies in the future, in addition to increased pollen production and extended flowering seasons (Ziska and Beggs 2012).

It has also been reported that elevated CO₂ increased photosynthetic activity, leading to changes in the leaf nonstructural carbohydrates (sugars and starches) with an increase on average by 30–40 % per unit leaf area (Ainsworth 2008; Ainsworth and Long 2005; Taub 2010). However, on our pollen experiments, the exposure to CO₂ just led to statistically significant changes in the fructose content, but only when exposition time was of 6 h, which could point out to a minor influence of CO₂ on the pollen-soluble carbohydrate content. Also, these results may indicate that the decrease in *A. negundo* pollen viability and germination observed after exposure to CO₂ do not seem to be directly related with changes in carbohydrate content. It is suggested that the type of carbohydrate reserves influence the mean life of pollen (Pacini 1996). Mature pollen grains have different types of carbohydrates, being glucose, fructose, and sucrose the most frequent sugars found (Clement and Pacini 2001). Sucrose seems to influence pollen longevity by stabilizing the plasma membranes, keeping it viable during dehydration and transport (reviewed in Pacini 1996), and in some Poaceae species, viable pollen presented higher fructose and glucose content compared with less viable one. Nonetheless, in our experiments, a relation between pollen fertility decrease and carbohydrate content after CO₂ exposure could not be established.

To sum up, in our study, it was verified that increasing concentrations of CO₂ and exposure times can alter *A. negundo* airborne pollen fertility, total soluble protein content, allergenicity, and fructose content. No significant changes were observed in the pollen-soluble carbohydrates such as sucrose and glucose.

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