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► **To cite this version:**

Nicolas Visez, Mona Hamzé, Klervi Vandebossche, Florent Ocelli, Patricia de Nadaï, et al.. Uptake of ozone by allergenic pollen grains. *Environmental Pollution*, 2023, 331, pp.121793. <10.1016/j.envpol.2023.121793>. <hal-04115098>

HAL Id: hal-04115098

<https://hal.science/hal-04115098v1>

Submitted on 9 Jul 2025

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Uptake of Ozone by Allergenic Pollen Grains

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Keywords: Air Pollution, Allergy, Atmospheric Biochemistry, Bioaerosol, Oxidative Stress

Abstract

Ozone exacerbates allergy symptoms to certain pollens. The molecular mechanisms by which ozone affects pollen grains (PGs) and allergies are not fully understood, especially as the effects of pollutants may vary depending on the type of pollen. In this work, pollens of 22 different taxa were exposed under laboratory conditions to ozone (100 ppb) to quantify the ozone uptake by the PGs. The ozone uptake was highly variable among the 22 taxa tested. The highest ozone uptake per PG was measured on *Acer negundo* PGs (2.5 ± 0.2 pg.PG⁻¹). On average, tree pollens captured significantly more ozone than herbaceous pollens (average values of 0.5 and 0.02 pg.PG⁻¹, respectively). No single parameter (such as the number of apertures, time of the year for the pollen season, pollen size, or lipid fraction) could predict a pollen's ability to take up ozone. Lipids seem to act as a barrier to ozone uptake and play a

32 protective role for some taxa. After inhalation of PGs, pollen-transported ozone could
33 be transferred to mucous membranes and exacerbate symptoms through oxidative
34 stress and local inflammation. Although the amount of ozone transported is small in
35 absolute terms, it is significant compared to the antioxidant capacity of nasal mucus
36 at a microscale. This mechanism of pollen-induced oxidative stress could explain the
37 aggravation of allergic symptoms during ozone pollution episodes.

38 1 Introduction

39 The pollen matrix is composed of a myriad of bioactive molecules that are co-
40 delivered with allergens (Visez et al., 2021). A growing body of evidence supports an
41 essential role of these bioactive molecules during allergic sensitization. It is
42 hypothesized that these bioactive adjuvants contribute to the creation of a pro-
43 inflammatory microenvironment at the cellular level upon exposure to a pollen grain
44 (PG); a microenvironment that would induce dendritic cells to promote Th2
45 lymphocyte polarization (Pointner et al., 2020). The pollen matrix is at the source of
46 the chemical microenvironment of pollen in contact with mucosal cells, which can be
47 modified by the chemical aging of the PG.

48 The PG is notably modified by gaseous air pollutants, in particular by SO₂, NO₂ and
49 O₃; these three gases have been widely used in laboratory experiments of PGs
50 fumigation (Frank and Ernst, 2016; Sénéchal et al., 2015; Wolters and Martens,
51 1987). Ground-level ozone concentrations during the 21st century are likely to remain
52 at levels hazardous to plant health (Sicard et al., 2017). Weather conditions favorable
53 to pollination, such as calm, sunny weather, are also conducive to ozone formation. In
54 Copenhagen, for example, high ozone concentrations coincide with both the birch
55 and grass pollen seasons and the daily pollen peaks (Ørby et al., 2015). There is also a
56 coincidence between the grass pollen season and the highest ozone concentrations
57 (Bastl et al., 2021). Furthermore, atmospheric concentrations of allergenic PGs are
58 likely to increase for some taxa in the coming decades, especially under the combined
59 influence of climate change and increasing atmospheric CO₂ concentrations (Barnes,
60 2018). Therefore, studying the effects of ozone on PGs is more relevant than ever in a
61 context where the prevalence of pollen allergy is estimated at 40% in Europe
62 (D'amato et al., 2007). This is especially important because ozone increases the
63 severity of allergy symptoms (Berger et al., 2020).

64 Little is known about the molecular mechanisms involved in the effects of ozone on
65 pollen allergy, and further research is needed in this area. The effects of ozone on
66 pollen germination were first reported by Feder in 1968 (Feder, 1968). Since then, a
67 series of effects following exposure of pollen of many taxa to ozone have been
68 described: decreased viability (Cuinica et al., 2013b, 2013a; Pereira et al., 2021;
69 Wolters and Martens, 1987); partial or complete inhibition of pollen tube formation
70 (Chichiricco and Picozzi, 2007; Cuinica et al., 2013b, 2013a; Feder, 1986, 1981, 1968;
71 Ferreira et al., 2015; Gillespie et al., 2015; Harrison and Feder, 1974; Leisner and
72 Ainsworth, 2012; Masaru et al., 1976; Roshchina and Mel'nikova, 2001; Wolters and
73 Martens, 1987); chemical modification of sporopollenin (Dominguez et al., 1999;
74 Goschnick and Schuricht, 1996; Naas et al., 2016; Roshchina and Mel'nikova, 2001);
75 modification of the lipidic fraction (Beck et al., 2013; Farah et al., 2020b; Naas et al.,
76 2016; Zhu et al., 2018); promotion of protein nitration reactions or modification of
77 some proteins after simultaneous exposure of pollen to ozone and nitrogen dioxide
78 (Estillore et al., 2016; Franze et al., 2005; Galveias et al., 2020; Hong et al., 2018; Liu
79 et al., 2017; Meusel et al., 2017; Ribeiro et al., 2017); increase or decrease of the total
80 amount of extractable proteins and allergens (Albertine et al., 2014; Cuinica et al.,
81 2013b, 2013a; Eckl-Dorna et al., 2010; Ferreira et al., 2015; Masuch et al., 1997;
82 Ribeiro et al., 2014, 2013); change in allergenicity (IgE reactivity to allergens and
83 ELISA tests) (Cuinica et al., 2015; Eckl-Dorna et al., 2010; Lu et al., 2020; Ribeiro et
84 al., 2014); larger wheal and flares of skin prick test for pollen harvested from trees
85 with higher ozone exposure (Beck et al., 2013); higher propensity to rupture
86 (Rogerieux et al., 2007); enhancement of the activity of the enzyme NAD(P)H oxidase
87 (Pasqualini et al., 2011); change in the microbial composition of pollen (Obersteiner
88 et al., 2016).

89 Existing research already clearly indicates that the effects of ozone are dose- and
90 species-dependent, and in most cases these effects result in increased allergenicity
91 (Frank and Ernst, 2016). A diversity of responses to environmental stresses is indeed
92 expected depending on the species, including both the plant parts and the pollen or
93 its allergens (Feder, 1986, 1981; Frank and Ernst, 2016; Shahali and Dadar, 2018;
94 Wolters and Martens, 1987). It is challenging to compare the results of pollen
95 exposures to pollutants carried out under different experimental conditions given
96 that several parameters vary from one study to another, including the ratio between
97 the mass of pollen treated and the mass of pollutant used, the concentration of

98 pollutant, the contact time, and the control of relative humidity and the hydration
99 rate of the pollen. However, some studies have shown that under strictly identical
100 experimental conditions, pollens of different taxa react differently to exposure to
101 atmospheric oxidants, albeit on a limited number of taxa (between 3 and 7 per study)
102 (Gute et al., 2020; Pereira et al., 2021; Ribeiro et al., 2013; Roshchina and
103 Mel'nikova, 2001). The effect of pollutants is also sometimes completely opposite, for
104 example an increased IgE reactivity to proteins of *Acer negundo* and *Quercus robur*
105 pollen in response to ozone treatment, while the opposite was observed for *Platanus*
106 × *acerifolia* pollen under the same experimental conditions (Ribeiro et al., 2014).

107 The sensitivity of pollen to ozone clearly varies between species, and also between
108 exposure conditions (Wolters and Martens, 1987). Therefore, it is difficult to estimate
109 whether one or more species are more sensitive to ozone while others are more
110 resistant. In this work, we exposed the pollen of 22 taxa to ozone under strictly
111 identical conditions to quantify the ozone uptake by these different species. Contact
112 between pollen and ozone in the laboratory can involve ozone consumption through
113 two mechanisms: adsorption of ozone on the surface of PGs and reactivity of ozone
114 with PGs constituents (Alebić-Juretić et al., 1997). Adsorbed ozone can be carried on
115 the PG and transferred to the mucous membranes after inhalation of the PG. Pollen-
116 borne ozone could be involved in aggravation of symptoms by oxidative stress and
117 local inflammation.

118 2 Materials and methods

119 2.1 Pollen samples

120 Commercial pollen samples were all provided by BONAPOL (České Budějovice,
121 Czechia), with the exception of one batch of *Ambrosia artemisiifolia* provided by
122 Greer Laboratories (Lenoir, USA), and batches of *Pinus halepensis* and *Cupressus*
123 *sempervirens* collected harvested respectively in Algeria and France. BONAPOL
124 pollens were dried immediately after the collection to the water content <7% and
125 stored at 5 °C for 6 months and then at -18 °C.

126 A multi-annual series of 5 batches of birch pollen was also collected from 2016 to
127 2020 on the campus of the University of Lille, Faculty of Sciences and Technologies.
128 A multi-location series of 9 batches of *Betula pendula* pollen collected in 2020 from 9
129 different sites in Croatia (3 sites) and Czechia (6 sites) was also provided by

130 BONAPOL. The onset of pollination was estimated from the French national
131 aerobiology network data (RNSA: www.pollens.fr). Pollen size and mass data were
132 taken from the literature (Guerin, 1993).

133 The water content was measured for some pollens (*Betula pendula*, *Alnus glutinosa*,
134 *Salix caprea*, *Urtica dioica*, *Fraxinus excelsior*, *Pinus halepensis* and *Cupressus*
135 *sempervirens*) by weight difference after storage of the pollen in a drying oven heated
136 to 110 °C for 48 hours. The water content of all tested pollens was lower than 2%,
137 consistent with the collection protocol that involved drying the samples and storing
138 them in airtight vials at 4 °C.

139 2.2 Determination of the lipid fraction

140 A 500 mg sample of pollen was placed in a Pasteur pipette and washed with
141 dichloromethane (10 mL). After the solvent was drained, the pollen was dried under
142 nitrogen flow to obtain a defatted pollen batch. The solvent from the washing step
143 was evaporated to dryness under nitrogen flow; the mass of the dry residue was
144 obtained by weighing (± 0.1 mg). The average mass of extractable lipids was obtained
145 from three samples of 500 mg each. It should be noted that the internal lipids of
146 some taxa are not extracted by the dichloromethane wash on intact (uncrushed)
147 pollen (Farah et al., 2020a).

148 2.3 Measurement of the specific surface area

149 Specific surface area was measured by adsorption followed by desorption of N₂(g) at
150 77 K on 300 mg pollen samples (Flowsorb III Micromeritics®). The gas stream used
151 was a N₂/He mixture with a ratio of 30/70. Prior to the measurement, the pollen
152 samples were degassed at room temperature for 30 minutes under the same N₂/He
153 gas stream.

154 The pollen specific surface area ($\mu\text{m}^2 \cdot \text{PG}^{-1}$) was calculated from the measured specific
155 surface area ($\text{m}^2 \cdot \text{g}^{-1}$) and the number of PGs per milligram of pollen obtained from
156 literature (Guerin, 1993). The geometric surface area of a PG was calculated using the
157 surface area of a sphere with the same diameter.

158 2.4 Ozone uptake

159 The experimental setup for pollen exposure to ozone has been described elsewhere
160 (Zhu et al., 2018). Briefly, an ozone stream passed through a filter holder containing a
161 47 mm quartz filter. Once the ozone concentration was stable (100 ± 5 ppb), 5 ± 0.1 mg

162 of pollen was quickly introduced into the filter holder and exposed to ozone. The
163 ozone concentration has been measured every 10 s by an analyzer (Thermo Scientific
164 model 49i) placed at the outlet of the filter holder. When ozone uptake occurred after
165 pollen introduction, a negative peak of ozone was observed. The integration of this
166 peak gives the mass of ozone uptaken per mg. Each measurement was repeated three
167 times for each pollen taxon. The maximum deviation from the mean value of the
168 three measurements uncertainty was considered as the uncertainty. The error
169 margins were calculated as the maximum deviation among the ozone uptake values
170 from the mean of the measurements. We consider this method of ozone uptake
171 measurement to be sensitive for ozone masses greater than 1 ng per milligram of
172 pollen.

173 2.5 Statistical analysis

174 Differences in ozone uptake between tree and herb pollens were explored using a
175 Mann-Whitney test. To test the influence of pollen features on ozone uptake, Mann-
176 Whitney and Kruskal-Wallis tests were respectively conducted for two-groups and
177 three-groups comparisons. Correlation between pollen size, lipid fraction and ozone
178 uptake were evaluated using the Spearman's correlation coefficient. The threshold for
179 statistical significance was set to $p < 0.05$. Statistical analyses were carried out using
180 XLSTAT statistical software.

181 3 Results

182 3.1 General overview

183 The good repeatability of ozone concentration measurements is demonstrated on four
184 replicates of ozone exposure of *Olea europaea* pollen (Figure 1). Upon introduction of
185 the pollen, the ozone concentration drops abruptly and then gradually rises to a level
186 close to the initial concentration.

187 The ozone uptake measurements were conducted in ng per mg of pollen and then
188 converted to uptaken ozone mass per pollen grain in pg per PG (Table 1). The pollens
189 tested in this work can be classified into two categories based on their ozone uptake
190 levels: those with undetectable ozone uptake (< 0.05 pg.PG⁻¹) and those with
191 significant ozone uptake (> 0.1 pg.PG⁻¹). The highest ozone uptake per PG was
192 observed on *Acer negundo* pollen (2.5 ± 0.2 pg.PG⁻¹). A statistically significant

193 difference was observed between trees (n=13) and grasses (n=8), with a mean of 0.5
194 and 0.02 pg.PG⁻¹, respectively (p-value = 0.032 < 0.05).

195 3.2 Within species variability for several batches of *Betula pendula* pollen

196 The ozone uptake by *Betula pendula* pollen was tested on 14 different pollen batches:
197 5 multi-year batches for the same site and 9 multi-site batches for the same year
198 (Table 2). The amount of ozone captured by *Betula pendula* pollen ranged from 16 to
199 37 ng per mg of pollen (average 28±12 ng.mg⁻¹).

200 3.3 Role of lipids in the uptake of ozone

201 Ozone uptake was measured for several taxa after defatting, which involves washing
202 the pollen with an organic solvent to remove the outer lipid layer (Table 3). Pollen
203 without its external lipids tended to uptake a much larger amount of ozone for *Fagus*
204 *sylvatica*, *Pinus halepensis* and *Ambrosia artemisiifolia*. However, the effect of
205 defatting on ozone uptake in birch pollen is not conclusive between our study and
206 that of Zhu et al. (2018). We believe that changes in the experimental protocol may
207 explain this difference. The ozone uptake values reported by Zhu et al. (2018) were
208 obtained for pollen samples defatted several days before ozone exposure, whereas
209 ozone uptake measurements were performed in this work directly after pollen
210 extraction and drying.

211 The ozone uptake per pollen grain was plotted as a function of the external lipid mass
212 for both untreated and defatted pollen in Figure 2. Three groups of pollen can be
213 distinguished on this graph: pollen with low ozone uptake (<0.2 pg.PG⁻¹)
214 independently of the lipid fraction, pollen with high ozone uptake (> 0.2 pg.PG⁻¹),
215 and finally defatted pollen. A positive statistical correlation is observed between
216 ozone uptake (for non-defatted pollen) and the lipid fraction for ozone uptakes higher
217 than 0.2 pg.PG⁻¹ ($R_s = 0.599 > 0.5$).

218 3.4 Specific surface area of pollen

219 The pollen specific surface (μm².PG⁻¹) is directly proportional ($R^2=0.993$) to the
220 geometric surface of the PGs (Table S1). On average, the specific surface of pollen is
221 approximately four times larger than its geometric surface. The specific surface of
222 untreated pollen is low, less than 1.3 m².g⁻¹ for all taxa tested in this work. Pollen
223 defatting increased the specific surface of *Ambrosia artemisiifolia* pollen but did not
224 significantly change that of *Betula pendula*, *Fagus sylvatica* and *Pinus halepensis*

225 pollens (Table 3). Figure 3 illustrates the diversity of ozone uptakes measured for
226 some taxa as a function of pollen specific surface ($\mu\text{m}^2.\text{PG}^{-1}$). There is no clear
227 correlation between pollen surface area and ozone uptake, with pine pollen being an
228 emblematic example of a taxon with the highest surface area of all tested taxa but
229 relatively low ozone uptake.

230 3.5 Influence of pollen characteristics on ozone uptake

231 3.5.1 Apertures

232 The hypothesis tested is whether apertures, which are thinner or missing parts of the
233 exine through which the pollen tube can develop, may represent weak points in the
234 protection of pollen from gaseous pollutants. Statistical analyses show that the
235 number of apertures does not seem to influence the rate of ozone uptake ($\text{pg}.\text{PG}^{-1}$) by
236 pollen (p-value = 0.66). However, most of the pollen tested (16 out of 22 taxa) had
237 three apertures, so further ozone uptake experiments using pollen grains with a
238 greater diversity of aperture numbers are needed to confirm this result. Additionally,
239 the type of aperture (porate, colpate, colporate, etc.) was not found to be statistically
240 correlated with ozone uptake.

241 3.5.2 Pollen season

242 We hypothesized here that taxa with a summer pollination season would be adapted
243 to the frequent high ozone concentrations with lower uptake rates. Statistical analysis
244 showed that the rate of ozone uptake by pollen grain ($\text{pg}.\text{PG}^{-1}$) is not correlated to the
245 pollination period (winter/spring/summer) (p-value = 0.34).

246 3.5.3 Pollen size

247 The Spearman correlation coefficient indicates a weak positive correlation between
248 pollen size and the mass of ozone uptaken by the PGs ($r_s = 0.34$). However, this weak
249 statistical correlation should not overshadow the significant differences between taxa.
250 For instance, the largest pollen in our series, namely pine, exhibits very low ozone
251 uptake.

252 4 Discussion

253 4.1 Ozone uptake by pollen

254 In this study, we have demonstrated that some pollen grains (PGs) can uptake ozone
255 under laboratory conditions, while others do not. In our study, we found that there
256 was a great diversity of ozone uptake among the 22 different taxa tested.

257 Interestingly, we observed that tree pollens on average had a higher ozone uptake
258 than herbaceous pollens. We could not identify a single parameter that would predict
259 the ability of a pollen to uptake ozone. It is likely that one of the key parameters
260 involved in ozone uptake is probably the chemical composition of the PG surface,
261 including both the chemical composition of the surface lipids and the molecular
262 structure of the sporopollenin. Previous studies have shown that the sensitivity of
263 pollen to ozone is dependent on its pigmentation, which is linked to its composition
264 in carotenoids and phenolic pigments such as flavonoids, anthocyanins and
265 coumarins (Roshchina and Mel'nikova, 2001). Pollen has been found to be an
266 effective adsorbent for certain gases or organic compounds, as reported in previous
267 studies (Okuyama et al., 2007; Thio et al., 2011; Zhu et al., 2018). In fact, PGs and
268 pollen-derived substances such as sporopollenin have been used for the detoxification
269 of environmental pollutants, particularly in aqueous media (Yaacob et al., 2022).

270 For pollens with an ozone uptake higher than 0.2 pg.PG^{-1} , lipids seem to play a role in
271 the interaction of pollen with O_3 . A pre-treatment consisting in washing the pollen
272 with a solvent to remove external lipids (defatting) strongly increased ozone uptake
273 in the cases of *Fagus sylvatica*, *Pinus halepensis* and *Ambrosia artemisiifolia*.
274 Defatting also has a marked effect in increasing the pollen specific surface area of
275 *Ambrosia artemisiifolia* pollen. We suggest a double role of lipids towards ozone
276 uptake. Firstly, the presence of lipids can promote adsorption and reactivity with
277 ozone. The reactivity of lipids with a double bond towards ozone has already been
278 described for three taxa: *Betula pendula*, *Phleum pratense* and *Pinus halepensis*
279 (Farah et al., 2020b; Naas et al., 2016; Zhu et al., 2018). On the other hand, lipids can
280 limit ozone uptake on the pollen surface by physically blocking access to certain
281 channels or microcavities (hence reducing the pollen specific surface area).
282 Therefore, lipids have a protective role for pollen against ozone, either by antioxidant
283 activity (especially via lipids with double bonds), or as a layer physically protecting
284 the underlying pollen surface.

285 Laboratory ozone uptake is not an absolute indicator of the susceptibility of a PG to
286 ozone. Firstly, ozone can react slowly and without an absorption step. As example,
287 pine pollen has a low ozone uptake (2 ng.mg^{-1}), and yet reactivity has been observed
288 between ozone and some pine pollen lipids (unsaturated fatty acids), as well as with
289 coumaric acid monomers on the sporopollenin exine surface (Naas et al., 2016). A

290 low ozone uptake indicates that ozone will not react or be absorbed on the pollen
291 surface; however, reactivity by an Eley–Rideal mechanism, where ozone reacts
292 directly with compounds on the surface of the PG from the gas phase without passing
293 through an adsorption step, cannot be excluded.

294 4.2 Effects of pollen-borne ozone on nasal inflammation

295 Unravelling the interactions of PGs with nasal cells is an emerging research question
296 that challenges our understanding of the molecular mechanisms at the cellular level,
297 as well as the biochemical composition of PGs, including pollution-induced changes.
298 The amounts of ozone uptaken at the scale of a single PG are extremely low in
299 absolute value, with a maximum amount measured in this work at 2.6 picograms of
300 ozone uptaken per PG of *Acer negundo*. Is this low dose of ozone sufficient to disrupt
301 the protective role of nasal mucus and cause damage to the underlying cells? Ozone
302 may indeed cause oxidative stress in the respiratory track if not scavenged by
303 antioxidants before reaching epithelial cells (Pöschl and Shiraiwa, 2015). To test this
304 hypothesis, it is necessary to compare the quantity of ozone brought on the epithelial
305 surface during the deposition of a PG with the quantity of ozone in the gas phase
306 captured by the same epithelial surface. To do so, we will consider a single PG
307 deposition in the nasal cavity (Horváth et al., 2011). We assume the deposition of one
308 PG of *Acer negundo* on a $35 \times 35 \mu\text{m}$ surface of the nasal epithelium (pollen diameter
309 $\sim 35 \mu\text{m}$), which could provide a maximum ozone transfer to the mucus of 2.6 pg for
310 one deposited grain (Table 1). Knowing that about a quarter of inhaled ozone gas is
311 eliminated in the nasal cavity (Keshavarzi, 2011), the amount of ozone gas deposited
312 on a $35 \times 35 \mu\text{m}$ of epithelial surface can be estimated considering a single breath
313 volume of 0.5 L, an ozone concentration of $200 \mu\text{g}\cdot\text{m}^{-3}$ (equivalent to 100 ppb) and a
314 nasal mucosa surface of 160 cm^2 (Gizurarson, 2012). With these values, the average
315 ozone concentration taken up by this surface area of $1225 \mu\text{m}^2$ ($35 \times 35 \mu\text{m}$) is
316 estimated to be 7.7 fg, i.e. an amount of ozone more than 300 times lower than the
317 one delivered by a PG of *Acer negundo* on the same surface. Therefore, the amount of
318 ozone transported by a PG represents a significant dose for the nasal epithelium
319 compared to the gaseous ozone input. To calculate the amount of ozone transferred
320 from the pollen grain to the mucus, we assumed that the ozone captured by the pollen
321 grain is fully available (with no reactivity or irreversible adsorption). The fate of
322 ozone after being captured on the pollen surface was not studied in this work.
323 Previous research by Zhu et al. (2018) suggests that only a fraction of the adsorbed

324 ozone reacts with alkenes on the surface of birch pollen. Additional measurements
325 are needed to accurately quantify the fraction of available ozone.

326 It is also interesting to compare the ozone dose delivered by a PG with the
327 antioxidant capacity of nasal mucus. In the upper airways, the dose of ozone reaching
328 the tissues is generally low because ozone reacts with mucosal compounds. This is
329 due to the mucus layer that protects the underlying cells, including the reactivity of
330 ozone with antioxidants (Medinsky and Bond, 2001; Pöschl and Shiraiwa, 2015).
331 However, if pollen delivers a high dose of ozone locally, it is conceivable that the
332 natural protection offered by the nasal mucus is exceeded, and ozone can reach nasal
333 cells at higher doses (Figure 4). This tissue damage could cause additional or
334 preparatory inflammation to the release of allergens and adjuvants by pollen. This
335 extends the oxidative stress hypothesis (Naclerio et al., 2020) in the sense that a
336 pollutant is concentrated and transported on the surface of a PG, implying a higher
337 dose of oxidant during the deposition of a single PG on the cell surface than that
338 received from the gas phase. The mechanism of oxidative stress induced by pollen-
339 borne ozone could explain the aggravation of symptoms during co-exposure to pollen
340 and ozone.

341 The concentrations of the two main antioxidants in mucus are about 250 μM for uric
342 acid and 30 μM for ascorbate (Pöschl and Shiraiwa, 2015). Using these
343 concentrations, we can estimate the amount of antioxidants present in a mucosal
344 volume of $35 \times 35 \times 10 \mu\text{m}^3$, i.e. 3.1 and 0.4 femtomole for uric acid and ascorbate,
345 respectively. It is interesting to note that the mass of ozone delivered in the gas phase
346 (7.7 fg) in the same volume during one breath corresponds to 0.2 femtomoles, which
347 is one to two orders of magnitude less than the cumulative antioxidant capacity of
348 uric acid and ascorbate. In contrast, the amount of pollen-borne ozone (2.6 pg.PG^{-1})
349 corresponds to 54 fmol, which is an order of magnitude greater than the local
350 antioxidant capacity. The comparison between these quantities of matter shows that
351 the hypothesis of overwhelming the local antioxidant capacity (3.5 fmol) by pollen-
352 borne ozone (54 fmol.PG^{-1}) is plausible, while the amount of ozone brought by the air
353 is not sufficient (0.2 fmol per breath at an ozone concentration of $200 \mu\text{g.m}^{-3}$ / 100
354 ppb) to alter this oxidant capacity. The phenomena involved are undoubtedly
355 complex, and our calculations of stoichiometric proportions are only intended to
356 provide orders of magnitude to test the hypothesis of overwhelmed anti-oxidant

357 capacity due to pollen-borne ozone. The reactivity of ozone on the surface of PGs is
358 not taken into account in these calculations. Ozone can be consumed at the surface of
359 pollen through reactions with unsaturated lipids (Naas et al., 2016; Zhu et al., 2018),
360 which can also generate reactive species such as Reactive Oxygen Species and
361 Reactive Oxygen Intermediates that contribute to the oxidative capacity of pollen
362 (Shiraiwa et al., 2011). Experimental investigations at the scale of the PG are needed
363 to understand the reactivity at its surface and its potential implications on the
364 epithelial cell inflammation, especially as other pollutants adsorbed on the pollen
365 surface may also affect the antioxidant activity of nasal mucus. In fact, the reactivity
366 of epithelial antioxidants, including uric acid and ascorbic acid, decreases by several
367 orders of magnitude when the pH locally drops from 7 to 3 (Pöschl and Shiraiwa,
368 2015). The mucus may be locally acidified by the deposition of airborne particles
369 inhaled simultaneously with pollen (Pöschl and Shiraiwa, 2015). Pollen can also carry
370 acidic compounds such as HNO_2 , HNO_3 , HCl (Chassard et al., 2015; Okuyama et al.,
371 2007) as well as atmospheric particles that can locally modify the microenvironment
372 at the pollen deposition site (Choël et al., 2022; Visez et al., 2020).

373 There is a significant body of consistent information from real-world studies on co-
374 exposure to pollen and pollution to incriminates air pollutants in the aggravation of
375 pollen allergy symptoms (Berger et al., 2021; Naclerio et al., 2020; Stas et al., 2021).
376 For instance, high allergic symptom severity scores were linked to exposure to birch
377 pollen concentrations greater than 100 PG.m^{-3} and exposure to ozone and PM_{10} with
378 concentrations greater than $10 \mu\text{g.m}^{-3}$ (Stas et al., 2021). Ozone was also positively
379 and significantly associated with symptom scores during the grass pollen season, but
380 not for those of birch and ragweed (Berger et al., 2020). The study by Makra et al
381 (2014) highlighted the role of pollen and chemical air pollutants in hospital
382 admissions for respiratory symptoms. They demonstrated that atmospheric exposure
383 to O_3 and pollens (other than ragweed) were the first and second risk factors,
384 respectively, for elderly people (>65 years) with allergic rhinitis (Makra et al., 2014).
385 This association was not found in younger adults (<65 years), and the difference can
386 be explained by greater dysfunction of the epithelial barrier in the elderly. Murine
387 models of co-exposure to ozone and non-pollen allergen show an increase in the
388 number of epithelial cells and the appearance of mucus cells in areas that are
389 normally free of these secretory cells after three days of co-exposure (Wagner et al.,
390 2002). In humans, ozone exposure does not promote the early phase of the allergen-

391 induced inflammatory response, but it does increase the influx of eosinophils
392 involved in the late phase of the allergic reaction after exposure to the allergen
393 (Michelson et al., 1999). All of this data supports significant dysfunction at the nasal
394 level, which leads to an aggravation of allergic inflammation induced by co-exposure
395 to O₃ and allergens.

396 To our knowledge, the hypothesis that pollen-transported oxidative stress causes
397 aggravated health effects has not yet been tested in laboratory studies involving
398 exposure to either pollen extracts or allergenic proteins alone. The transport of
399 pollutants on the pollen surface is indeed not being tested when only allergenic pollen
400 extracts are inhaled. For example, the broncho-constrictive response for allergens
401 alone and co-exposure to ozone and allergens was not statistically different for birch
402 and grasses (Ørby et al., 2019). This mechanism of pollutant transport can only be
403 significant with atmospheric particles having a high surface area per particle for
404 ozone adsorption. Thus, the transported dose is much higher than the local
405 antioxidant capacities at the site of adhesion of the particle on the epithelial surface.
406 To our knowledge, this mechanism of pollutant transport by pollen has not been
407 proposed elsewhere. Studies on the effects of ozone on the PG have mainly focused on
408 reactivity, including the modification of lipids, proteins, allergens or the pollen
409 surface. However, there are specific conditions that need to be followed to study the
410 mechanism of pollen-transported oxidative stress in laboratory conditions. The
411 lifetime of ozone or its derivatives (reactive oxygen species, reaction products) on the
412 pollen surface is not known. Under real exposure conditions, an equilibrium is
413 reached between the concentration of free gaseous ozone and the adsorbed quantity.
414 Based on our ozone uptake experiments, the characteristic time to reach this
415 equilibrium should be of the order of several tens of minutes for pollens with high
416 ozone uptake. Under real life conditions, pollen is inhaled under these equilibrium
417 conditions, and the transported ozone can reach the cellular level. In laboratory
418 experiments, ozone pollution of pollen is often temporally dissociated from cell
419 exposure to polluted pollen. Therefore, it is conceivable that the ozone uptaken on the
420 pollen is eliminated during pollen storage pending cell exposure. Dedicated
421 experimental protocols are necessary to consider the mechanism of pollen-
422 transported oxidative stress. This involves exposing cells to pollen that has been
423 freshly exposed to ozone as well as pollen that has been exposed to ozone long
424 enough to reach a steady state in terms of the amount of pollutant transported. Air-

425 liquid interface studies with nasal cells that specifically address these points are
426 needed to better understand the biochemistry of pollen interaction with epithelial
427 cells.

428 5 Conclusion

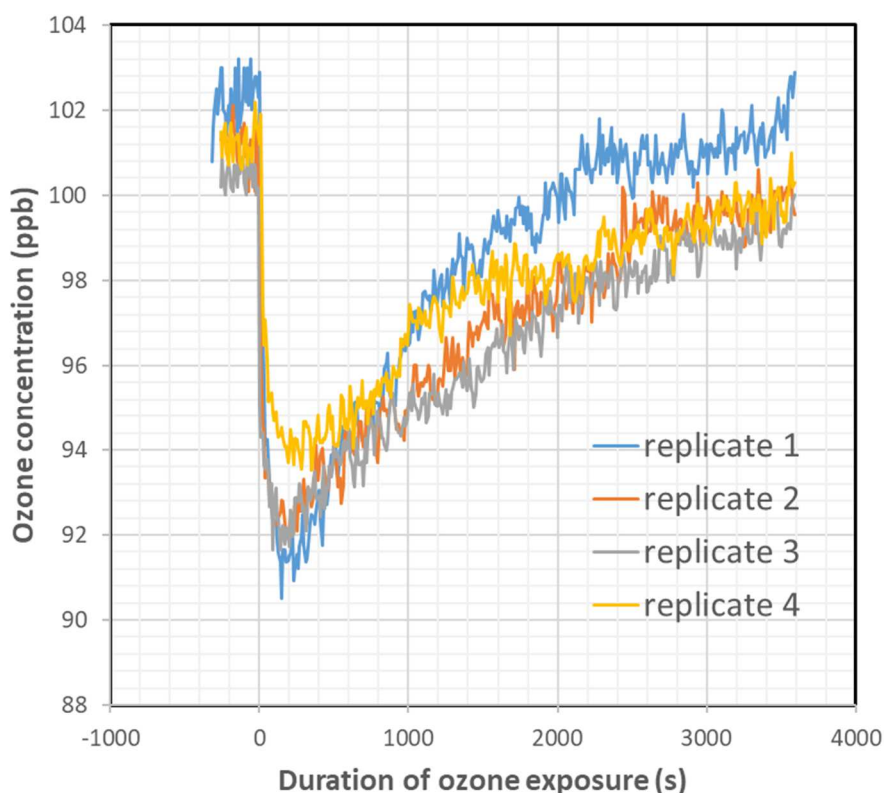
429 Ozone uptake was measured on 22 different pollen taxa using an experimental setup
430 that allowed for the measurement of minimal ozone uptake in the nanogram per
431 milligram range with excellent repeatability (approximately $\pm 10\%$). The same
432 experimental setup was used to measure ozone uptake in 14 different birch pollen
433 samples from different locations or years of harvest, and the reproducibility was
434 good, with an average ozone uptake by birch pollen of 28 ± 12 ng.mg⁻¹. No single factor
435 was found to explain why some pollens uptake ozone while others do not. The taxon-
436 specific quantitative and qualitative properties of the surface lipid fraction, as well as
437 the structure of exine, probably play a crucial role in the ozone uptake and reactivity.
438 This work highlights the importance of not extrapolating the effects of pollutants
439 from one pollen taxon to another.

440 Ozone uptake by pollen may play a role in both the integrity of the epithelial barrier
441 and in local inflammation upon contact with the nasal epithelium. The dose of ozone
442 carried by pollen can locally exceed the antioxidant capacities, supporting the pollen-
443 transported oxidative stress hypothesis. Some pollens with the highest ozone uptake
444 are the best candidates for future studies on the aggravation of inflammation and
445 allergic symptoms during co-exposure to ozone. These include *Acer negundo*, *Olea*
446 *europaea*, *Phleum pratense*, *Fraxinus excelsior* and *Corylus avellana*.

447 When deposited on mucous membranes, pollen can modify its surrounding
448 microenvironment with a multitude of molecules constituting the PG. This
449 microenvironment may also be modified depending on the aging processes
450 undergone by the PG in the atmosphere, such as the uptake of gaseous oxidants,
451 deposition of atmospheric particles, or even rupture of the pollen grain due to
452 climatic conditions and mechanical constraints. A better understanding of the state of
453 PG at the time of inhalation is essential to comprehend the molecular and
454 biochemical mechanisms by which human health is affected by pollen.

455 6 Acknowledgements

456 NV thanks Olivier Gardoll (Unité de Catalyse et de Chimie du Solide, Univ. Lille) for
457 his help for specific surface area measurements. The authors thank the University of
458 Lille and the Institut de Recherches Pluridisciplinaires en Sciences de
459 l'Environnement (IREPSE Fed 4129) for financial support. The CaPPA project
460 (Chemical and Physical Properties of the Atmosphere) is funded by the French
461 National Research Agency (ANR) through the PIA (Programme d'Investissement
462 d'Avenir) under contract ANR-11-LABX-005-01. The authors also thank the French
463 Ministère de l'Enseignement Supérieur et de la Recherche, ADEME, the Hauts de
464 France Region and the European Funds for Regional Economic Development for
465 their financial support to this project. The authors thank the Re´gion Hauts-de-
466 France, the Ministe`re de l'Enseignement Supe´rieur et de la Recherche and the
467 European Fund for Regional Economic Development for their financial support to the
468 CPER CLIMIBIO and ECRIN programs.



469
470 Figure 1. Repeatability of ozone uptake with *Olea europaea* pollen monitored at the
471 reactor outlet. Each data trace represents a different experiment of exposure of 5 mg
472 of pollen to 100 ppb of ozone, with a total of four independent samples (n = 4). The
473 introduction of pollen into the ozone stream is marked as t=0 s. The decrease in
474 ozone concentration indicates an ozone mass captured by the pollen of $130 \pm 12 \text{ ng} \cdot \text{mg}^{-1}$,
475 equivalent to $1.6 \pm 0.2 \text{ pg} \cdot \text{PG}^{-1}$.

476 Table 1. Mass of ozone uptaken per mg of pollen and per pollen grain (PG). The
 477 uncertainty is the maximum deviation of the three measurements from the mean.
 478 The different taxa are described from literature data (Guerin, 1993; Halbritter et al.,
 479 2018): start of pollination, number of apertures, pollen size and mass, number of
 480 pollen grains per mg, year of harvest (supplier data). The lipid mass was obtained by
 481 extraction with dichloromethane (see Section 1.2).

| | O ₃ capture per mg (ng.mg ⁻¹) | O ₃ capture per PG (pg.PG ⁻¹) | Harvest Year | Pollination (month of year) | Number of pollen apertures | Lipids (mg.g ⁻¹) | Pollen size (μm) | Pollen mass (ng) | ×10 ³ PGs per mg |
|---|--|--|-----------------|-----------------------------------|----------------------------------|---------------------------------|----------------------------|------------------------|--------------------------------|
| trees | | | | | | | (Values from Guérin, 1993) | | |
| <i>Acer negundo</i> | 82 ±7 | 2,5 ±0.2 | 2018 | 4 | 3 | 51 | 35 | 30 | 33 |
| <i>Olea europaea</i> | 130 ±12 | 1,6 ±0.2 | 2017 | 5 | 3 | 74 | 27 | 12 | 83 |
| <i>Fraxinus excelsior</i> | 50 ±4 | 0,65 ±0.05 | 2018 | 3 | 3 | 19 | 27 | 13 | 77 |
| <i>Quercus rubra</i> | 25 ±3 | 0,45 ±0.05 | 2018 | 4 | 3 | 20 | 30 | 18 | 56 |
| <i>Corylus avellana</i> | 34 ±2 | 0,34 ±0.02 | 2018 | 1 | 3 | 21 | 25 | 10 | 100 |
| <i>Fagus sylvatica</i> | <1 | <0.05 | 2016 | 4 | 3 | 18 | 43 | 50 | 20 |
| <i>Cupressus sempervirens</i> | 18 ±2 | 0,28 ±0.03 | 2009 | 2 | 0 | 14 | 28 | 15 | 67 |
| <i>Pinus halepensis</i> | 2 ±2 | 0,2 ±0.2 | 2014 | 4 | 1 | 17 | 50 | 90 | 11 |
| <i>Betula pendula</i> | 37 ±1 | 0,23 ±0.01 | 2019 | 3 | 3 | 27 | 21 | 6 | 160 |
| <i>Alnus glutinosa</i> | 11 ±2 | 0,10 ±0.02 | 2017 | 1 | 5 | 34 | 24 | 9 | 110 |
| <i>Populus nigra</i> | <1 ±1 | <0.02 | 2016 | 3 | 0 | 30 | 29 | 16 | 63 |
| <i>Salix caprea</i> | <1 ±1 | <0.01 | 2007 | 3 | 3 | 39 | 23 | 8 | 125 |
| <i>Platanus x hispanica</i> | <1 ±0 | <0.01 | 2011 | 4 | 3 | 39 | 20 | 5 | 200 |
| herbs | | | | | | | | | |
| <i>Parietaria officinalis</i> | 109 ±2 | 0,11 ±0.01 | 2017 | 5 | 3 | 0 | 10 | 1 | 1000 |
| <i>Urtica dioica</i> | 66 ±2 | 0,07 ±0.01 | 2017 | 6 | 3 | 43 | 10 | 1 | 1000 |
| <i>Ambrosia artemisiifolia (Bonapoli)</i> | <1 | <0.01 | 2012 | 7 | 3 | 118 | 25 | 10 | 100 |
| <i>Chrysanthemum leucanthemum</i> | <1 | <0.01 | 2017 | 6 | 3 | 49 | 28 | 14 | 71 |
| <i>Rumex acetosella</i> | 6 ±2 | 0,01 ±0.01 | 2018 | 5 | 3 | 43 | 20 | 5 | 200 |
| <i>Ambrosia artemisiifolia (Greer)</i> | <1 | <0.01 | n/a | 8 | 3 | 120 | 25 | 10 | 100 |
| <i>Artemisia vulgaris</i> | <1 | <0.01 | 2018 | 7 | 3 | 25 | 21 | 6 | 167 |
| <i>Plantago lanceolata</i> | <1 | <0.01 | 2018 | 5 | >6 | 15 | 25 | 10 | 100 |
| grass | | | | | | | | | |
| <i>Phleum pratense</i> | 15 ±2 | 0,50 ±0.07 | 2018 | 5 | 1 | 22 | 35 | 33 | 30 |

n/a: not available

482

483

484 Table 2. Ozone uptake measurements of different *Betula pendula* pollen batches
 485 collected (i) from the same site for 5 consecutive pollen seasons (2016 to 2020); (ii) at
 486 9 different locations during the 2020 pollen season (batches provided by BONAPOL).

| Pollen batch | Harvest Year | O ₃ capture per mg (ng.mg ⁻¹) | O ₃ capture per PG (pg.PG ⁻¹) |
|--|--------------|--|--|
| Different years with the same harvesting location | | | |
| Univ. Lille | 2016 | 29 ±3 | 0.18 ±0.02 |
| Univ. Lille | 2017 | 19 ±3 | 0.12 ±0.02 |
| Univ. Lille | 2018 | 29 ±3 | 0.18 ±0.02 |
| Univ. Lille | 2019 | 37 ±3 | 0.23 ±0.02 |
| Univ. Lille | 2020 | 35 ±2 | 0.22 ±0.01 |
| <i>average</i> | | 30 ±11 | 0.19 ±0.07 |
| Different locations harvested in 2020 | | | |
| Croatia, Zagreb region (a) | 2020 | 36 ±4 | 0.23 ±0.03 |
| Croatia, Zagreb region (b) | 2020 | 22 ±2 | 0.14 ±0.01 |
| Croatia, Zagreb region (c) | 2020 | 30 ±1 | 0.19 ±0.01 |
| Czechia, South Bohemia (a) | 2020 | 26 ±1 | 0.16 ±0.01 |
| Czechia, South Bohemia (b) | 2020 | 32 ±2 | 0.20 ±0.01 |
| Czechia, South Bohemia (c) | 2020 | 16 ±2 | 0.10 ±0.01 |
| Czechia, South Moravia (a) | 2020 | 16 ±2 | 0.10 ±0.01 |
| Czechia, South Moravia (b) | 2020 | 32 ±1 | 0.20 ±0.01 |
| Czechia, South Moravia (c) | 2020 | 26 ±2 | 0.16 ±0.01 |
| <i>average</i> | | 26 ±10 | 0.16 ±0.07 |

487

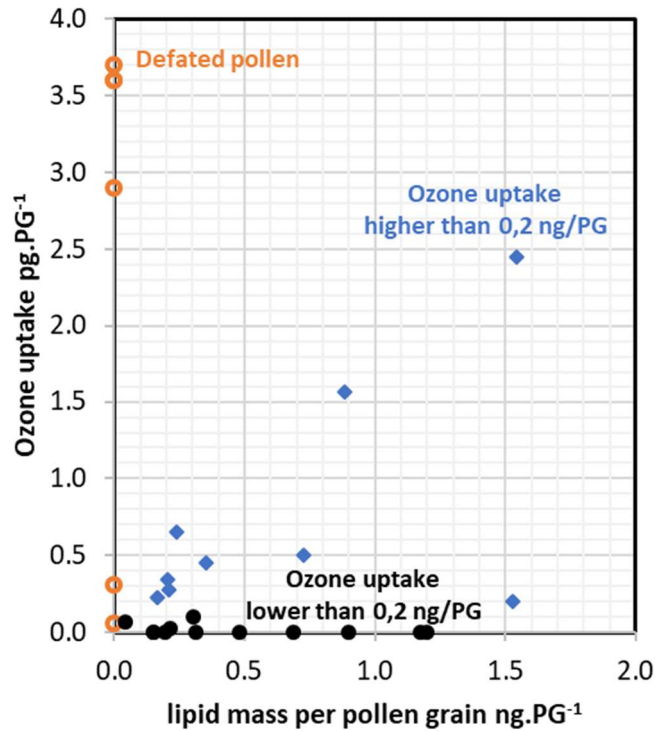
488

489 Table 3. Comparison of ozone uptake (pg per pollen grain) between untreated pollen
 490 and dichloromethane-treated (defatted) pollen.

| | O ₃ capture | | lipids for intact PG ng.PG ⁻¹ | specific area m ² /g | specific area for defatted pollen m ² /g | |
|------------------------------|----------------------------|------------------------------|--|---------------------------------|---|-----------------|
| | intact pg.PG ⁻¹ | defatted pg.PG ⁻¹ | | | | |
| <i>Betula pendula</i> | 0.18±0.02 | 0.06±0.02 | 0.2 | <i>n/a.</i> | <i>n/a.</i> | Zhu et al. 2018 |
| <i>Betula pendula</i> | 0.23±0.01 | 0.31±0.01 | 0.2 | 0.4 | 0.4 | this work |
| <i>Fagus sylvatica</i> | <1 | 3.6±0.1 | 0.9 | 0.3 | 0.3 | this work |
| <i>Pinus halepensis</i> | 0.2±0.2 | 3.7±0.4 | 1.5 | 1.2 | 1.3 | this work |
| <i>Ambrosia a. (BONAPOL)</i> | <0.01 | 3.6±0.8 | 1.2 | 0.6 | 2.3 | this work |
| <i>Ambrosia a. (Greer)</i> | <0.01 | 2.9±0.9 | 1.2 | 0.6 | 3.1 | this work |

n/a.: not available

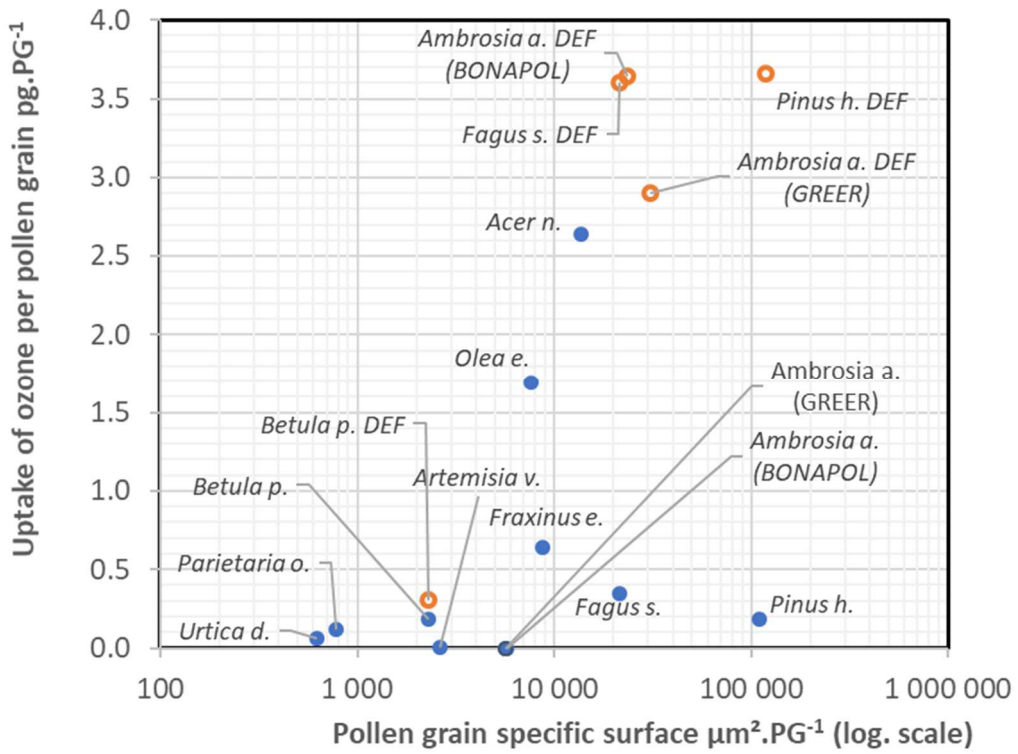
491



492

493 Figure 2. Uptake of ozone (pg per PG) as a function of the external lipid mass (ng per
 494 PG). Three groups of pollens can be distinguished: defatted pollens with significant
 495 ozone uptake (orange circles), pollens with negligible ozone uptake (<0.2 pg.PG⁻¹)
 496 irrespective of the external lipid mass (black dots) and finally pollens with ozone
 497 uptake >0.2 ng.PG⁻¹ (blue diamonds).

498

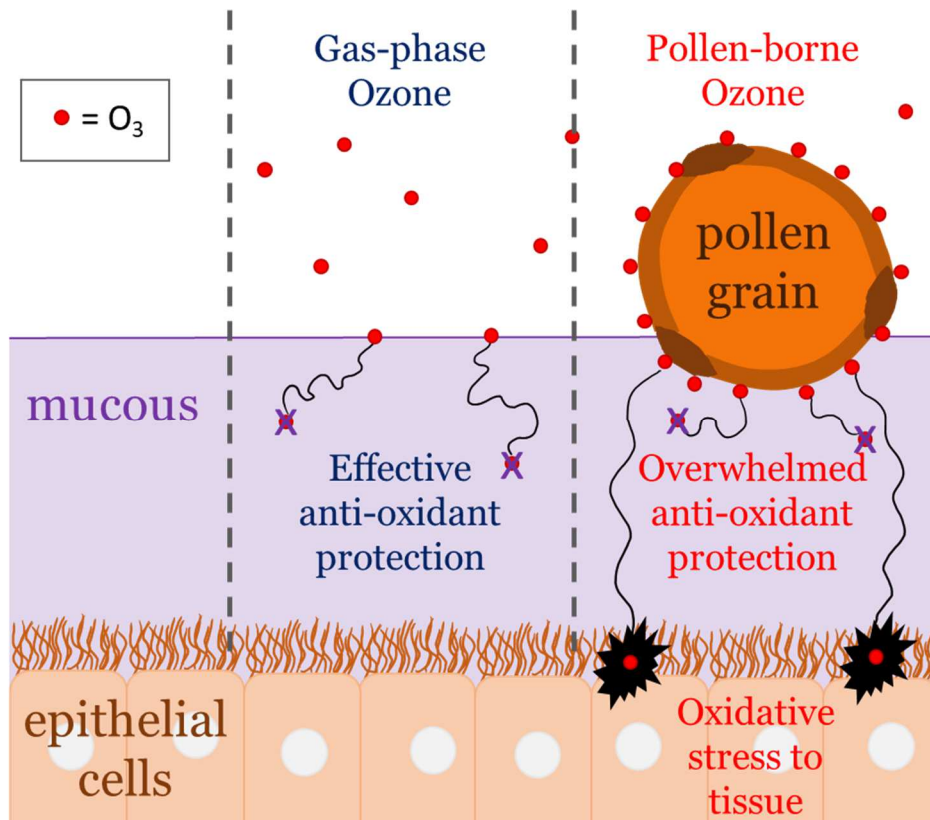


500

501 Figure 3. Ozone uptake per pollen grain (pg.PG⁻¹) as a function of the specific surface
 502 area expressed per pollen grain (µm².PG⁻¹). Solid symbols correspond to untreated
 503 pollens and open symbols to defatted pollens (DEF).

504

505



506

507 Figure 4. Schematic representation of a proposed mechanism for the overwhelming
 508 of antioxidant defenses in nasal mucus. The amount of ozone uptaken on a pollen
 509 grain (up to 2.6 pg.PG⁻¹) is locally high in the nasal epithelium. This local high dose
 510 could exceed the antioxidant capacity of the mucus and cause cellular damage. The
 511 dose of ozone delivered to the mucus by the uptake of ozone from the gas phase is
 512 effectively annihilated by the antioxidant species in the nasal mucus (uric acid and
 513 ascorbate). Mucus thickness is exaggerated in the diagram for clarity (actual
 514 maximum thickness: 10 μm).

515

516

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