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Exposure of *Betula pendula* Roth pollen to atmospheric pollutants CO, O₃ and SO₂

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Abstract

Betula pendula pollen, under laboratory conditions, was exposed to three atmospheric pollutants: carbon monoxide (CO), ozone (O₃) and sulphur dioxide (SO₂). Two levels of each pollutant were used; the first level corresponds to a concentration on the atmospheric hour-limit value acceptable for human health protection in Europe, the second level to a higher, at least more than double of the first, concentration level. Experiments were done under artificial solar light with controlled temperature and relative humidity. Our results indicate that, in urban areas, concentrations of CO, O₃ and SO₂ on the limits established for human protection, can affect pollen fertility. We verified a decrease in the viability and germination of the pollen, indicating damage to the pollen membrane system. Also, a general decreasing trend in the total protein content of the exposed samples when compared with the control samples was observed, which suggests alterations in the antigenic characteristics of pollen.

Keywords: *Betula* pollen, atmospheric pollutants, pollen viability, pollen germination, protein content

Pollen is a natural atmospheric environmental allergenic in several cases responsible for severe human health effects (Linskens & Cresti, 2000; Traidl-Hoffmann et al., 2009; d'Amato et al., 2010; Bosch-Cano et al., 2011). In the atmosphere, pollen co-exists with air pollutants emitted from anthropogenic sources such as carbon monoxide (CO) resulting from incomplete combustion of carbon materials, ozone (O₃), a secondary pollutant generated in photochemical smog, and sulphur dioxide (SO₂) emitted during burning of fossil fuels. The direct effect of air pollutants on pollen and on its allergenic potential is currently an important scientific subject. However, the effect of air pollutants on the allergenic potential of pollen is not yet completely clear, but it has been suggested that it contributes to increase allergic sensitisation (Traidl-Hoffmann

et al., 2009; Bosch-Cano et al., 2011; Sousa et al., 2012).

Atmospheric pollutants interact with pollen causing changes on its fertility affecting the reproductive cycle of seed plants (Bell & Treshow, 2002; Omasa et al., 2002; Elagoz & Manning, 2005). At cellular level, air pollutants provoke damage of the membrane structures and interfere with cellular mechanisms as well as in the gene expression (Koch et al., 1998; Roshchina & Roshchina, 2003; Kangasjärvi et al., 2005). There is some evidence, but not confirmed experimentally, that the pollen wall contains antioxidants such as carotenoids that trap reactive oxygen atoms and reduce the negative potential of air pollutants (Castillo et al., 2005).

The adverse effects of air pollutants on fertility are usually detected during pollen germination

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(Feder & Sullivan, 1969). Taking these observations into consideration, Chichiriccò and Picozzi (2007) raised the hypotheses that the oxidative properties of the air pollutants should provoke the inactivation of the germination factors present in pollen. In terms of metabolism, respiration is the fundamental factor for germination owing to energy produced (White et al., 1968; Brown & Borutaite, 2004). The air pollutant CO has affinity towards the mitochondrial cytochromes in being a potential inhibitor of the cellular respiration (Brown & Borutaite, 2004). Nitrogen dioxide (NO₂), O₃ and their reactive specific derivatives (superoxides, hydroxyl radicals, peroxides and nitrogen containing reactive molecules) provoke damage of the cellular membrane, and O₃ may even cause cellular organelle disconnection (Black et al., 2000; Wheeler et al., 2001; Omasa et al., 2002).

At molecular level, air pollutants have a relatively strong oxidative role that affects biomolecules such as proteins, lipids and nucleic acids that constitute the main material pollen reservoir, interfering with pollen germination and elongation of the pollen tube (Harrison & Feder, 1974; Chichiriccò, 2000; Roshchina & Mel'nikova, 2001; Omasa et al., 2002).

Betula trees (Betulaceae) are extensively planted for ornamental purposes in parks and gardens in the urban area of Porto. They are anemophilous, producing great amounts of pollen and thus are commonly recorded in aerobiological and allergenic studies. Their pollen is a common source of pollinosis in Europe (d'Amato et al., 2007). However, sensitisation to pollen allergens is variable between different regions of the world, according to climate, urbanisation levels, geography and vegetation (du Bay & Murdy, 1983).

Table I. Average and standard deviation of the concentration of CO, O₃ and SO₂, temperature and relative humidity during the *Betula pendula* pollen exposure experiments.

Pollutant	Experiment	Concentration (ppm)	Temperature (°C)	Relative humidity (%)
CO	+	9.77 (1.28)	27 (1)	71 (1)
CO	++1d	30.98 (3.97)	27 (1)	68 (1)
CO	++2d	23.05 (3.38)	27 (1)	65 (1)
O ₃	+	0.061 (0.017)	27 (1)	70 (1)
O ₃	++1d	0.192 (0.026)	27 (1)	66 (1)
O ₃	++2d	0.189 (0.036)	27 (1)	72 (2)
SO ₂	+	0.19 (0.05)	27 (1)	57 (1)
SO ₂	++1d	0.35 (0.20)	29 (1)	56 (1)
SO ₂	++2d	0.54 (0.24)	29 (1)	57 (1)

Standard deviations in parenthesis. Symbols represent: +, about the hour-limit value; ++1d, about two times the hour-limit value during one day; ++2d, about two times the hour-limit value during two days.

The aim of this research was to study the effects of CO, O₃ and SO₂ on viability, germination and protein content of *Betula pendula* Roth pollen. This objective was achieved by exposing pollen samples under controlled experimental conditions at two different gas concentration values: just below the safety standard limits for human health protection and to a higher level corresponding roughly to about the double of the first level during one and two days.

Material and methods

Pollen samples

During the flowering season, male catkins of *Betula pendula* were collected from the botanical garden and the city park of Porto and dried at 27 °C. After two days, the catkins were gently crushed and passed through different grades of sieves to obtain pure pollen, which was then stored at –20 °C until use.

In vitro exposure to pollutants

Pollen samples were artificially exposed in an environmental chamber (Sousa et al., 2012). The chamber is made of a mix of acrylic and wood with dimensions about 50 cm × 70 cm × 50 cm. Sunlight in the chamber 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. A fan (SUNON SF23080AF) was used to homogenise 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.

Each pollen sample [300 mg of dry weight (DW)] was exposed individually to CO, O₃ and SO₂ under two different concentrations: The first concentration level corresponds to the current atmospheric hour-limit value acceptable for human health protection in Europe (8.73 ppm for CO, 0.061 ppm for O₃ and 0.13 ppm for SO₂; European Union Directive 2008/50/EC of 21 May 2008 on ambient air quality and cleaner air for Europe) during a 6h assay; the second level, at least more than the double of the first concentration level (this higher level was used to confirm experimentally any effect on the pollen that could be observed upon exposure to the lower level). The six hours exposure time was used as a model for the period of time corresponding to the highest solar irradiation (around noon) and the hours with the highest traffic in a town including the morning rush hour – the period of time corresponding

to the highest air pollutants concentration. Sulphur dioxide (99.9% v/v) concentrations were attained by injecting gas directly obtained from bottles of compressed gas (Sigma-Aldrich). Carbon monoxide was generated by chemical reaction of sulphuric acid with formic acid by mixing, in a test tube, stoichiometric amounts of the two concentrated acids and subsequently injecting it into the chamber. Three injections of gas per assay were performed using a micro syringe within each two hour interval in order to prevent greater concentration fluctuations. Ozone was generated using an A2Z OZONE system coupled with an OMRON H3DK-S1 timer, controlling the injection of O₃ for brief periods of time in order to offset the high O₃ reactivity. Non-exposed pollen samples were used as control. Pollen samples were exposed to controlled pollutant levels and environmental conditions (Table I).

Pollen viability

Pollen viability was estimated using the Trypan blue stain, a vital dye. Its reactivity is based on the fact that it does not interact with the cell unless the membrane is damaged. Thus, all non-coloured pollen are viable. Pollen grains (2 mg) were placed for 5 min in 0.5 ml of 60% Trypan blue with subsequent centrifugation for 3 min at 13 200 rpm. Then 20 µl of the residue were placed on a microscope slide and observed with a Leica DMLB light microscope. Pollen viability was determined as percentage of non-coloured pollen relative to the total pollen. For each sample (exposed and non-exposed pollen), two slides were prepared and for each slide, 600 pollen grains were counted.

In vitro pollen germination

Non-exposed and exposed pollen samples were germinated at 27 °C in the dark for 24 hours in a germination medium optimised by the authors (200 ppm H₃BO₃, 400 ppm L-proline, 15% sucrose, 0.5% agar). Two replicates were performed and in each, five random fields were counted (100 pollen grains per field). A pollen grain was classified as germinated when the tube was longer than the pollen size (Nepi & Franchi, 2000).

Protein extraction and quantification

Pollen grains (50 mg) 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 four hours. The suspension was centrifuged at 13 200 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 (Bradford, 1976).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) in 12.5% (w/v) polyacrylamide gels under non-reducing conditions. The proteins were visualised by Coomassie Brilliant Blue R-250 staining. The molecular mass of polypeptide bands was estimated by comparison with protein markers (PageRuler Plus Prestained Protein Ladder, Fermentas).

Results

Pollen viability

The analyses of the *Betula pendula* pollen viability show that pollen viability decreases after the exposure to the pollutant gases. Non-exposed pollen shows a viability of 93 ± 1%, whilst exposed pollen, at the highest concentration, had a viability of about 80% (Table II). An important result is that, even at the lowest pollutant level, which is below the safety standard limit for human health, a decrease in pollen viability was observed. The analysis of variance (ANOVA) of the effect of the pollutant exposure on pollen viability reveals a statistical significance (Table III).

Table II. Results of the viability, germination and total soluble protein (TSP) content of *Betula pendula* pollen exposed to CO, O₃, and SO₂.

Pollutant	Experiment	Viability (%)	Germination (%)	TSP (µg/ml)
Control	C	93 (1)	39 (1)	1238 (355)
CO	+	89 (2)	31 (2)	843 (12)
CO	++1d	84 (1)	27 (1)	1004 (63)
CO	++2d	82 (1)	25 (1)	869 (24)
O ₃	+	87 (1)	28 (2)	1218 (45)
O ₃	++1d	82 (2)	26 (1)	898 (17)
O ₃	++2d	78 (1)	25 (1)	927 (38)
SO ₂	+	87 (1)	29 (2)	750 (28)
SO ₂	++1d	82 (1)	27 (2)	695 (28)
SO ₂	++2d	80 (1)	25 (1)	726 (24)

Standard deviations in parenthesis. Symbols represent +, about the hour-limit value; ++1d, about two times the hour-limit value during one day; ++2d, about two times the hour-limit value during two days.

In vitro pollen germination rates

The analyses of the *in vitro* germination rates of *Betula pendula* pollen show a decrease in germination rates after exposure to the pollutant gasses. Non-exposed pollen shows a percentage of germination of $39 \pm 1\%$, whilst the exposed pollen, at the highest concentration, shows germination percentages of about 25% (Table II). These results agree with the viability results. ANOVA of the effect of the pollutant exposure on the pollen germination reveals statistical significance (Table III).

Protein pollen content

The protein content of the *Betula pendula* pollen shows a general decreasing trend of exposed samples compared to the control sample (Table II). However, the one-way ANOVA of this effect shows that it is not significant for a 0.05 probability level with the exception of SO_2 ($F = 6.2 > F_{\text{crit}}$). Indeed, SO_2 was the pollutant that induced a consistent decrease on the protein content (750 ± 28 , 695 ± 28 and $726 \pm 24 \mu\text{g/ml}$, respectively) when compared with the control ($1238 \pm 355 \mu\text{g/ml}$). The exposure with the other two pollutants provoked only a small decrease in the pollen soluble protein content when compared with the control.

Although the protein content of the exposed pollen showed some variation relatively to the non-exposed pollen, no changes in the polypeptide profile between exposed and non-exposed pollen samples were observed (Figure 1). The electrophoretic profile revealed several polypeptide bands ranging from 70 to 15 kDa after the Coomassie staining of the gel. The polypeptide profiles of all the pollen samples showed five clusters of bands of about 70, 55, 35, 25 and 15 kDa.

Discussion

The exposure of the *Betula pendula* pollen to the air pollutant gasses CO , O_3 and SO_2 below the safety limit for human health standards significantly affected the fertility of the pollen. Indeed, the analysis of the viability and germination of the pollen after exposure show significant decreases: about 14% in viability (from 93%, control, to about 80%) and 36% in germination rate (from 39%, control, to about 25%), at the highest pollutant level. The viability and germination percentages of the pollen were also affected even for the lowest pollutant level studied: about 26% in germination rate (from 39%, control, to about 29%). The results agree with those obtained for *Crocus vernus* All. (Iridaceae) in which Chichiricò and Picozzi (2007) observed a 50% germination rate when pollen was independently

Table III. Effect of the *in vitro* exposure of *Betula pendula* to CO , O_3 and SO_2 given as F -values of the analysis of variance (ANOVA).

Property	df	F_{crit}	F		
			CO	O_3	SO_2
Germination	3/20	3.2	123.0	135.0	87.1
Viability	3/16	3.1	91.8	143.8	168.8
Protein	3/8	4.1	3.0	3.1	6.2

Note: df, degrees of freedom within and between groups; F , F -test value for one-way analysis of variance; F_{crit} , critical value of the F -distribution for probability level of 0.05.

exposed to 0.5 ppm CO and 0.3 ppm O_3 . Lower germination rates were achieved when the concentrations increased to 25 ppm CO and 0.5 ppm O_3 .

In another *in vitro* study, a 40–50% germination rate decrease was observed for *Nicotiana* sp. (tobacco) pollen when exposed to 0.1 ppm of O_3 for five hours and thirty minutes (Feder, 1968). The same conclusion was obtained in an *in vivo* study with *Lepidium virginicum* L. pollen, where a 50% germination rate decrease was observed when pollen was exposed to 0.6 ppm of SO_2 for three and eight hours during the flowering season (du Bay & Murdy, 1983). Moreover, when the same pollen was exposed to 0.7 ppm of SO_2 , a significant reduction of the viability and inhibition of the elongation of the pollen tube was observed.

The fertilisation analysis presented here revealed that even under CO , O_3 and SO_2 concentrations considered safe for human health, the viability and germination of *Betula pendula* pollen were affected. However, the effect of the three pollutants was similar probably because relatively small concentrations were used in the experiments. Indeed, previous studies have detected specific effects when pollen is exposed to pollutants with different chemical properties (Omasa et al., 2002; Gottardini et al., 2004).

In addition to affecting fertilisation, exposure to CO , O_3 and SO_2 also affects the total soluble proteins of the pollen, although this was only statistically significant for the exposure to SO_2 (Tables II, III). The corresponding polypeptide profiles of the aqueous pollen extracts (Figure 1) reveal no detectable differences among the exposed and non-exposed pollen samples. All electrophoretic profiles have several bands ranging from 70 to 15 kDa in common, and the density of the bands undergoes no alteration. This shows that the exposure of pollen to the air contaminants did not significantly modify the chemical properties of the aqueous pollen extracts, i.e. the water solubility and molecular weight of the soluble proteins (within the selectivity and sensitivity of the SDS-PAGE technique).

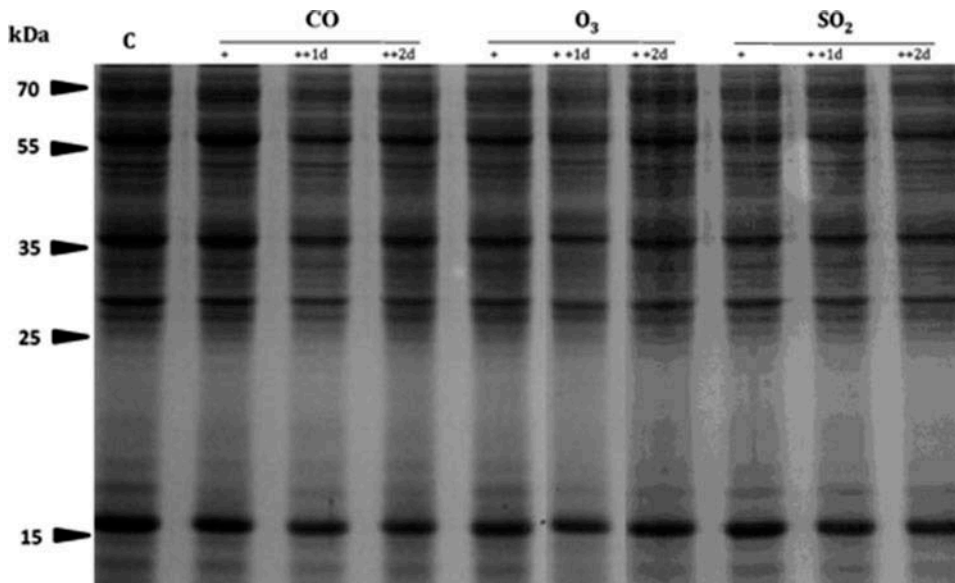


Figure 1. SDS-PAGE of soluble protein of *Betula pendula* pollen extracts exposed to CO, O₃ and SO₂. Symbols indicate gas concentrations levels: C, control; +, about the hour-limit value; ++1d, about two times the hour-limit value during one day; ++2d, about two times the hour-limit value during two days.

According to Sousa et al. (2012), atmospheric pollutants may increase or decrease the total protein content of pollen. Indeed, air pollution is a stress factor that affects the expression of proteins that are an integral part of the plant defence system as reported for the *Cupressus arizonica* Greene pollen in a high traffic area in Toledo, Spain (Cortegano et al, 2004). Similar results were observed for *Lagerstroemia indica* L. and *Spartium junceum* L. pollen exposed to SO₂ and CO in polluted areas of Teheran, Iran (Rezanejad et al., 2003; Rezanejad, 2007). In these studies, a reduction in the total soluble protein content was observed in the pollen when compared with samples from pristine areas (non-polluted). Another study about the exposure of *Argemone mexicana* L. pollen to 100 ppm of SO₂ showed that the total soluble protein content decreased as a consequence of the exposure, and that the values of decreasing increased with the time of the exposure from 24 to 48 hours (Parui et al., 1998). Other studies report the existence of unmeasurable differences in the polypeptide profiles of pollen extracts exposed and not-exposed to atmospheric pollutants (Rezanejad, 2007). The disappearance and intensity reduction of certain bands corresponding to soluble proteins of 22 and 45 kDa in the protein profile of *Canna indica* L. pollen exposed to the Teheran atmospheric pollution was referred by Majd et al. (2004). Rezanejad et al. (2003) also observed a significant decrease in the colouration intensity of several bands corresponding to soluble proteins when *Lagerstroemia* pollen was exposed to SO₂ and CO for ten and 20 days in polluted areas of Teheran. These data suggest that air pollution may indeed promote structural modifications in the proteins presents on the pollen wall (Chichiriccò & Picozzi, 2007).

The effect of air pollutants on the polypeptide profile of pollen is a subject that deserves further research. Indeed, the polypeptide profiles obtained in the present study are inconclusive, probably because the time of exposure of the pollen to the three pollutants was relatively short corresponding to only a single day of exposure. This may not be sufficient to provoke measurable changes on the polypeptide profiles when measured with SDS-PAGE. Further studies should focus on the concentration of the pollutants and exposure time in order to obtain information about the affected cellular mechanisms and the biochemical modifications on the proteins.

Conclusion

Betula pendula pollen, when exposed to the air pollutants CO, O₃ and SO₂ at levels that can be considered safe for human health protection, show significant negative effects on pollen viability and germination potential. Also, an apparent effect on the protein content of the exposed samples when compared with the control was detected, but this lacks a clear statistical signal. Taking into consideration that these results were obtained at concentration close to the limiting values for the protection of human health, this should raise further discussion about the existence of the synergistic effects between aerosol and air pollutants when threshold values for atmospheric pollutants are under consideration.

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