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Efeito de poluentes atmosféricos (CO, O_3, SO_2 e NO_2) na fertilidade e alergenicidade do pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus.*

Dissertação de candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Resumo

Nos últimos anos, tem-se verificado um aumento de doenças respiratórias, provocadas pelo pólen, em áreas urbanas e industrializadas. Este facto, pode estar relacionado com os níveis elevados de poluição atmosférica.

Neste trabalho, inicialmente otimizaram-se os meios de germinação *in vitro* para o pólen de Betulaceae de modo a serem usados ao longo do trabalho. Estudaram-se os efeitos dos poluentes - CO, O_3, SO_2 e NO_2 no pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus*, relativamente à fertilidade, proteínas solúveis, perfil de polipeptídeos e alergenicidade. Para cada gás testaram-se duas concentrações diferentes, ambas próximas das concentrações consideradas seguras para a proteção da saúde humana na Europa. A exposição do pólen a poluentes foi feita numa câmara de fumigação construída para o efeito, onde a luz solar foi simulada e a temperatura e humidade relativa foram monitorizadas. A viabilidade, germinação e conteúdo de proteínas solúveis do pólen exposto aos poluentes reduziram significativamente em comparação ao pólen não exposto. Os perfis dos polipeptídeos de todas as amostras de pólen, revelados por SDS-PAGE, apresentaram bandas entre 70 e 15 kDa. Não se observaram diferenças significativas entre o perfil de polipeptídeos das amostras do pólen exposto e não exposto. Os ensaios de imunodeteção mostraram maior reconhecimento de IgE em extratos proteicos do pólen exposto a poluentes em comparação com as amostras do pólen não exposto. A análise global mostra que o CO, O_3, SO_2 e NO_2 induziram maior reatividade de IgE de soros de pacientes em alergénios de extratos proteicos de pólen exposto a concentrações mais altas durante dois dias. As bandas reativas comuns nas três plantas, correspondem a proteínas de 58 e 17 kDa.

Estes resultados indicam que, concentrações de CO, O_3, SO_2 e NO_2 em valores-limite para proteção de saúde humana na Europa, podem agravar a polinose em pessoas sensibilizadas ao pólen de *Betula*, *Ostrya* e *Carpinus* bem como afetar a reprodução destas espécies arbóreas, dado verificar-se uma diminuição na fertilidade polínica.

Palavras-Chave: Sensibilização alérgica; Polinose; Immunoblotting; Fertilidade do pólen; Pólen de Betulaceae; Pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus Betulus*; Poluentes atmosféricos (CO, O_3, SO_2 e NO_2).

Abstract

In recent years, there has been an increase in respiratory diseases caused by pollen in urban and industrial areas. This may be related to high levels of pollution.

In this work, the media for the *in vitro* germination of Betulaceae pollen was optimized and the optimum conditions were used throughout the work. We studied the effects of air pollutants (CO, O_3, SO_2 and NO_2) in the pollen of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* and measured the fertility, soluble proteins, polypeptides and antigenicity profiles. For each gas two different concentrations were tested, both near the concentrations considered safe for human health protection in Europe. The exposure of pollen to pollutants was done on a fumigation chamber constructed for the purpose, where sunlight was simulated and the temperature and relative humidity were monitored. The viability, germination and soluble protein content of pollen exposed to pollutants were significantly reduced compared to non-exposed pollen. The profiles of the polypeptides of all pollen samples, revealed by SDS-PAGE, showed bands between 70 and 15 kDa. There were no significant differences between the polypeptide profiles of the pollen samples exposed and not exposed. Immunoblots showed increased IgE recognition of pollen protein extracts exposed to pollutants in comparison with the unexposed pollen samples. The analysis shows that CO, O_3, SO_2, and NO_2 induced higher serum IgE reactivity of patient allergens to pollen protein extracts exposed to higher concentrations for two days. Common reactive bands in all three plants correspond to proteins of 58 and 17 kDa.

These results indicate that concentrations of CO, O_3, SO_2 and NO_2 close to limit values used for protection of human health in Europe, may aggravate pollinosis in people sensitized to pollen from Betula, Ostrya and Carpinus, and affect the reproduction of tree species because a decrease in pollen fertility occurs.

Keywords: Allergic sensitization; pollinosis, Immunoblotting*;* Pollen fertility; Pollen of Betulaceae; *Betula pendula* pollen; *Ostrya carpinifolia* pollen; *Carpinus betulus* pollen; Atmospheric pollutants (CO, O_3, SO_2 and NO_2).

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Lista de Abreviaturas

ANOVA: Análise de variâncias

BSA (*bovine serum albumin*): albumina de soro bovino

CO: Monóxido de carbono

COV: Composto orgânico volátil

DW: Peso seco

ECL: Enhanced Chemiluminescence

IgE: Imunoglobulina E

kDa: *kilo Dalton*

NO_2: Dióxido de azoto

O_3: Ozono

PBS (*phosphate buffered saline*): Tampão fosfato salino

ppm: Partes por milhão

PVP: Poli-vinil-pirrolidona

rpm: Rotações por minuto

RT-PCR: (*Reverse Transcription – Polimerase Chain Reaction*): Transcrição reversa – Reação em cadeia de polimerase

SO_2: Dióxido de enxofre

SDS-PAGE (*Sodium dodecyl sulphate-polyacrylamide gel electrophoresis*): Electroforese em gel de poliacrilamida e dodecilsulfato de sódio

TSP: Proteína solúvel total

w/V: Massa/Volume

Lista de comunicações orais apresentadas em congressos científicos

1. **Effect of atmospheric pollutants CO, O_3, SO_2, and NO_2 in the *Betula* pollen** (IJUP 2013 – VI Encontro de Investigadores Jovens da Universidade do Porto).

2. **The effects of NO_2 exposure in the *Betula pendula, Ostrya carpinifolia* and *Carpinus betulus* pollen** (1st Iberian Meeting on Aerosol Science and Technology – RICTA 2013).

Capitulo I

Introdução

1. Introdução

1.1. Motivação

Em ambientes poluídos, o pólen é exposto a uma variedade de substâncias químicas, o que pode alterar não só a superficie exterior da parede do pólen bem como as proteínas associadas à alergenicidade. Este facto pode estar associado com o agravamento de sintomas alérgicos em indivíduos atópicos residentes em zonas urbanas e industriais (Wyler et al., 2000; Gilles et al., 2009; Traidl-Hoffmann et al., 2009; Bosch-Cano et al., 2011).

A prevalência de polinose tende a aumentar ao longo das duas últimas décadas, especialmente em países industrializados (*estimada em 40%*), mas as razões para este aumento ainda não são claramente conhecidas (Traidl-Hoffmann et al., 2009; D'Amato et al., 2010). Diversos estudos ambientais e epidemiológicos revelam que nos países da Comunidade Europeia, entre 8 e 35% dos adultos e jovens mostram maior reatividade de IgE à alergénios de pólen, estes casos são mais frequentes nos indivíduos residentes em áreas urbanas e industrializadas em relação aos que vivem em zonas rurais (D'Amato, 2000; Majd, 2004). O que leva a admitir que os poluentes presentes na atmosfera dos meios urbanos podem modificar a estrutura química das proteínas do pólen provocando aumentos significativos na resposta do IgE em indivíduos expostos (Emberlin, 1995; Devalia et al., 1998; Kim et al., 2013).

Equitativamente, este fenómeno pode fazer com que os poluentes atmosféricos provoquem alterações na fertilidade do pólen devido a forte atividade oxidativa que estas substâncias químicas têm de afetar as proteínas, lípidos e ácidos nucleicos (Bell e Treshow, 2002; Omasa et al., 2002; Elagoz e Manning, 2005). Isto pode estar associado a alteração do ciclo reprodutivo das plantas superiores.

Assim, este trabalho desenvolve um raciocínio analítico sobre a relação de causalidade entre os níveis de poluição atmosférica e o aumento de prevalência de alergias respiratórias causadas por pólen em zonas urbanas mais industrializadas, como um esforço de pesquisa preliminar para o

desenvolvimento de um novo conjunto de leis e normas lesgislativas a cerca da qualidade do ar na Europa.

1.2. Objetivos

O trabalho teve como objetivo principal, avaliar os efeitos de poluentes atmosféricos (CO, O_3, SO_2 e NO_2) na alergenicidade do pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus*, exposto *in vitro* a curta duração.

Assim, a pesquisa pretendeu desenvolver especificamente as seguintes tarefas:

- Encontrar melhor meio de germinação *in vitro* do pólen de plantas Betulaceae;
- Verificar os efeitos de CO, O_3, SO_2 e NO_2 nas taxas de viabilidade e germinação do pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus;*
- Verificar os efeitos de CO, O_3, SO_2 e NO_2 no conteúdo de proteínas solúveis do pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus;*
- Verificar os efeitos de CO, O_3, SO_2 e NO_2 no perfil electroforético dos polipeptídeos do pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus;*
- Analisar os efeitos de CO, O_3, SO_2 e NO_2 na alergenicidade do pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus*, usando soros de doentes alérgicos ao pólen de Betulaceae.

1.3. Legislação e padrões de qualidade do ar atmosférico na Europa

Tabela 1: Valores-limite de CO, O_3, SO_2 e NO_2

Poluente	µg/m³	ppm	Condições
CO	10000	8,73	Limite-Hora para prootecção da saúde humana
O_3	120	0,061	A não exceder mais de 25 dias num ano (*media anual*)
SO_2	350	0,13	Numa hora, a não exceder mais de 24 vezes num ano
NO_2	200	0,11	Numa hora, a não exceder mais de 18 vezes num ano

A tabela 1 ilustra as concentrações limite-hora de CO, O_3, SO_2 e NO_2 conforme a lei em vigor (*Directiva da União Europeia 2008/50/CE, de 21 de Maio de 2008 sobre a qualidade do ar ambiente e um ar mais limpo para a Europa*). Estes padrões e objetivos são aplicados sobre diferentes períodos de tempo, porque os impactos observados na saúde humana são associados a vários poluentes e ocorrem ao longo de diferentes tempos de exposição.

1.4. Estrutura da Dissertação

O trabalho apresenta a seguinte estruturação:

Primeiro Capítulo-***Introdução*** - Apresentam-se as razões que estimularam a realização desta pesquisa, os objetivos a atingir e a estrutura da dissertação.

Segundo Capítulo - ***Revisão Bibliográfica*** – Apresenta-se o estado de arte das diferentes temáticas abrangidas, fazendo uma breve resenha sobre a caraterização das espécies arbóreas tratadas no estudo (*Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus)* e o seu respetivo pólen, caraterização e toxicidade de CO, O_3, SO_2 e NO_2 no organismo humano e os efeitos destes poluentes atmosféricos no pólen, dando particular atenção à viabilidade, germinação, proteínas solúveis, perfil de polipeptídeos e alergenicidade.

Terceiro Capítulo - ***In vitro*** **germination of Betulaceae pollen** (*Trabalho enviado para publicação na revista "*Journal of Forest Research**")** - Apresenta-se os melhores meios de cultura para germinação *in vitro* do pólen das plantas de família Betulaceae.

Quarto Capítulo - **Efeitos de CO, O_3 e SO_2 na Viabilidade, Germinação, Conteúdo Proteico e Perfil de Polipeptídeos do Pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus*** - Neste capítulo faz-se uma análise dos efeitos de CO, O_3 e SO_2, em concentrações no limite e acima do limite permitido pela Lei em vigor, na viabilidade, germinação, conteúdo de proteinas solúveis e perfil de polipeptídeos do pólen de *Betula pendula, Ostrya carpinifolia* e *Carpinus betulus***.** Os resultados são apresentados em dois trabalhos: **Exposure of *Betula pendula* Roth pollen to atmospheric pollutants CO, O_3 and SO_2** (*Trabalho enviado para publicação na revista "*Grana*")* e ***In vitro*** **exposure of *Ostrya carpinifolia* and *Carpinus betulus* pollen to atmospheric levels of CO, O_3 and SO_2** (*Trabalho enviado para publicação na revista "*Environmental Science and Pollution Research*").*

Quinto Capítulo – (*Trabalho enviado para publicação na revista "*Ecotoxicology and Environmental Safety"), **Effect of air pollutant NO_2 on *Betula pendula*,**

***Ostrya carpinifolia* and *Carpinus betulus* pollen** - Neste capítulo analisam-se os efeitos de NO_2 em concentrações abaixo do limite permitido pela lei em vigor, na viabilidade, germinação, conteúdo de proteínas solúveis e perfil de polipeptídeos do pólen de *Betula pendula, Ostrya carpinifolia* e *Carpinus betulus*.

Sexto Capítulo - (*Trabalho enviado para publicação na revista "*Aerobiologia*")*, **Effects of atmospheric pollutants (CO, O_3, SO_2 and NO_2) on the allergenicity of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen** – Neste capítulo estudam-se os efeitos dos poluentes na alergenicidade do pólen de *Betula pendula, Ostrya carpinifolia* e *Carpinus betulus,* usando como anticorpo primário os soros de pacientes alérgicos ao pólen de Betulaceae.

Sétimo Capítulo – ***Conclusões e Perspectivas Futuras*** – Neste captítulo apresentam-se as principais conclusões de todos resultados obtidos, bem como sugestões para futuras investigações, sobre mecanismos das reações químicas promovidas por CO, O_3, SO_2 e NO_2 e outros poluentes atmosféricos nos alergénios dos grãos de pólen.

Capitulo II

Revisão Bibliográfica

2.1. Caraterização de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus*

Betula pendula, *Ostrya carpinifolia* e *Carpinus betulus* pertecem a família Betulaceae, um grupo de árvores de porte médio a grande com caraterísticas das regiões temperadas do hemisfério Norte, porém, algumas espécies atingem o hemisfério Sul nos Andes, na América do Sul (Chen et al., 1999). Estas plantas apresentam folhagem caduca, as folhas de disposição alterna são simples, estipuladas e serradas nas margens. As plantas são monóicas, ou seja, possuem flores masculinas e femininas separadas, mas na mesma planta, as flores masculinas formam-se em inflorescência estrobilóides pendentes ou eretas, laterais ou terminais (*Fig* 1). Os frutos são indeiscentes do tipo aquénio ou sâmara com asas curtas, uniloculares e monospérmicos (Chen et al., 1999).

Em zonas urbanas, estas árvores são usadas como plantas ornamentais, sendo plantadas em jardins e parques públicos. A *Betula pendula e Ostrya carpinifolia* também são economicamente importantes pela sua madeira resistente (Chen et al., 1999).

Fig 1: Inflorescência de *Betula* spp.

❖ Pólen

Pólen é o gametófito masculino produzido nas anteras das plantas superiores, e o seu principal papel é intervir na reprodução sexual de espermatófitas (Pérez et al., 2007). Esta estrutura biológica possui uma grande proporção de proteínas, uma variedade de aminoácidos e numerosas vitaminas (Puc e Wolski, 2002; Majd et al., 2004).

Betula pendula, *Ostrya carpinifolia* e *Carpinus betulus* possuem polinização anemófila, e o seu grão de pólen tem um diâmetro médio de 20 µm. Para uma fertilização bem-sucedida, as plantas anemófilas libertam grande quantidade de pólen aerodinâmico, para fácil dispersão e o aumento da probabilidade de fertilização (Esch et al., 2001; Traidl-Hoffmann et al., 2009). Estas caraterísticas, fazem com que este tipo de pólen seja mais associado ao surgimento de reações alérgicas respiratórias, por ser muito leve e por andar no ar geralmente de forma isolada (Esch et al., 2001). Assim, os alergénios libertados são frequentemente bem distribuídos no ar, e devido ao seu minúsculo tamanho, podem entrar facilmente em contacto com a via oral, nasal, ocular ou mucosa, induzindo o aparecimento de sintomas alérgicos no individuo (D'Amato et al., 2007; Bryce *et al.,* 2010).

A alergenicidade ao pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus* é muito frequente na Europa, especialmente em locais frescos e em zonas de climas temperados (D'Amato e Spieksma, 1991; Filon et al., 2000; Gumowski et al., 2000; Peternel et al., 2007; Çeter et al., 2012). No Norte da Europa os picos do pólen, são registados durante o mês de Maio, porém, no Sul a sua concentração aparece geralmente no início da primavera, atinge o pico em Abril e persiste até início do verão (D'Amato et al., 2007). Também, os casos de alergias ao pólen são mais registados no período de polinização das plantas (Bist et al., 2004).

2.2. Caraterização e toxicidade de CO, O_3, SO_2 e NO_2

a) Monóxido de Carbono

CO é um gás incolor, inodoro, levemente inflamável, e muito perigoso devido à sua grande toxicidade (Boian et al., 2006). É produzido maciçamente pela queima de carvão ou outros materiais ricos em carbono, como derivados de petróleo, em condições de pouco oxigénio e/ou alta temperatura (Masaru et al., 1976).

$$2C\,(s) + O_2\,(g) \rightarrow 2CO\,(g)$$

No organismo humano, o CO reduz a capacidade de transporte de oxigénio no sangue, por formar com a hemoglobina, um composto estável (carboxihemoglobina), deixando menos hemoglobina disponível para se ligar com

o oxigénio e fazer a sua transferência através do organismo, inibindo desta forma, a troca de oxigénio entre o sangue e os tecidos vitais (Sharan e Selvakumar, 1999). Este fenómeno pode causar danos temporários ou permanentes em tecidos que requerem grande quantidade de oxigénio para funcionar, induzindo dificuldades respiratórias, fadiga repentina, irritabilidade, tonturas, cefaleias, hiperventilação, disfunções no sistema nervoso central e problemas cardiovasculares em indivíduos com problemas coronários, anemia e alterações de hemoglobina mesmo a doses baixas (Sharan e Selvakumar, 1999). A exposição a concentrações elevadas ou até relativamente abaixo de 400 ppm do CO, pode provocar, em pessoas saudáveis, problemas de visão, dores de cabeça, redução da capacidade mecânica e de aprendizagem, dificuldade na resolução de certas tarefas e até mesmo provocar a morte (Joumard et al., 1983; Sharan e Selvakumar, 1999).

b) Ozono

O O_3 ostenta dois papéis contrários na atmosfera, pois, este gás é abundante na estratosfera e na troposfera. Na estratosfera forma uma camada com um papel bastante fundamental para qualquer vida no planeta terra, por absorver e reduzir a intensidade das radiações ultravioletas que tendem atingir a superfície do globo terrestre (Finlayson et al., 1986). Contudo, na troposfera (*baixa atmosfera*), o O_3 é um poluente ambiental, devido a instabilidade química da sua molécula, podendo destruir material orgânico, mecanismo pelo qual ataca o organismo humano e a vegetação (Manning e Godzik, 2004; Karlsson et al., 2007). Nesta região da atmosfera o O_3 é um dos principais oxidantes fotoquímicos e é responsável por até 90% de níveis totais de oxidantes em centros urbanos (McConnell et al., 2002).

O O_3 não é emitido diretamente para o ar, sendo formado na atmosfera, principalmente pela fotodissociação de óxidos de azoto na presença de compostos orgânicos voláteis (COVs) (Molfino et al., 1991).

$$NO_2(g) \xrightarrow{h\nu} NO(g) + O^{\cdot}(g) \qquad \lambda \text{ entre } 300 \text{ e } 400 \text{ nm}$$

$$O^{\cdot} + O_2(g) \xrightarrow{M} O_3(g) + M(g)$$

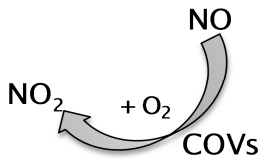
O O_3 formado neste processo seria rapidamente destruído por reação com o NO, o que não permitia a presença de grande concentração do O_3 na troposfera.

$$O_3\,(g) + NO\,(g) \rightarrow NO_2\,(g) + O_2\,(g)$$

Assim, o aumento de ozono troposférico é devido a contribuição ativa de outros oxidantes fotoquímicos formados a partir dos COVs, que possuem a capacidade de oxidar o NO em NO_2 sem consumir o O_3. Os oxidantes reagem com o NO, e este fica menos disponível para destruir o O_3, e os COVs aceleram a oxidação do NO em NO_2, que é dissociado pela radiação solar para formar o O_3. Este fenómeno favorece a formação excessiva de O_3 na troposfera, mesmo quando as concentrações dos seus precursores são extremamente reduzidas (Manning e Godzik, 2004).

$$NO_2\,(g) \xrightarrow{h\nu} NO\,(g) + O^{\bullet}\,(g) \qquad \lambda \text{ entre } 300 \text{ e } 400 \text{ nm}$$

$$O^{\bullet}\,(g) + O_2\,(g) \xrightarrow{M} O_3\,(g) + M$$



No organismo humano este poluente é inalado e absorvido pelas vias aéreas, penetra profundamente nas vias respiratórias, e afeta essencialmente os brônquios e os alvéolos pulmonares (Peden et al., 1995).

Mesmo em baixas concentrações e em exposições de curta duração, o O_3 pode causar aumento de permeabilidade epitelial no fluido broncoalveolar, podendo aumentar a suscetibilidade à infeções respiratórias, como disfunção pulmonar, redução da capacidade respiratória, inflamações brônquicas, pneumonia, dores no peito, tosse e náuseas (Horstman et al., 1995; Peden et al., 1995). Estes danos podem tornar-se permanentes em casos de exposições prolongadas ou repetidas, muito mais em grupos sensíveis, como crianças, idosos e indivíduos atópicos (McConnell et al., 2002).

A inalação de elevada concentração de O_3 pode induzir a deterioração da função pulmonar, aumento da reatividade das vias aéreas e agentes broncoconstritores

específicos, podendo aumentar significativamente os mediadores inflamatórios das células epiteliais brônquicas, e de fibronectina, o que pode agravar a saúde de indivíduos atópicos (Peden et al., 1995).

c) Dióxido de Enxofre

SO_2 é um gás incolor, não-inflamável e altamente tóxico. Este poluente é produzido e emitido para a atmosfera, naturalmente pelos vulcões e antropologicamente pela queima de carvão e de combustíveis fósseis (Carlson, 1983), bem como em certos processos industriais a partir da combustão de enxofre ou de pirite (FeS_2) (Varshney e Varshney, 1981).

$$S\,(s) + O_2\,(g) \rightarrow SO_2\,(g)$$

O SO_2 pode entrar no organismo humano pelo trato respiratório ou pela diluição na saliva, é inalado e absorvido no trato respiratório superior húmido e é rapidamente convertido em H_2SO_3 em contacto com as membranas das mucosas, podendo ser lentamente removido a partir do trato respiratório (Horstman et al., 1986). Depois da absorção na corrente sanguínea, o H_2SO_3 distribui-se amplamente por todo organismo, e é rapidamente convertido em SO_3^{2-} e HSO_3^{2-}, que por sua vez é oxidado a SO_4^{2-} e excretado na urina (Sheppard et al., 1980). Assim, em concentrações elevadas ou em situações de longa exposição, este poluente pode provocar efeitos irritantes, estimulando as extremidades nervosas presentes no revestimento das narinas, laringe e pulmão, causando tosse e uma sensação de aperto no peito, o que pode levar ao estreitamento das vias aéreas respiratórias e modificação de respostas imunes em indivíduos atópicos (Horstman et al., 1986).

Mesmo em concentrações muito baixas, o SO_2 pode induzir broncoconstrição aguda em indivíduos sensíveis (Sheppard et al., 1980). Ao contrário do O_3, o efeito broncoconstritor de $SO_{2,}$ em indivíduos atópicos, ocorre após períodos de exposição extremamente curtos, especialmente com a respiração oral em situações de alta ventilação, como é o caso da prática de exercícios físicos ao ar livre (D'Amato et al., 2010).

d) Dióxido de Azoto

NO_2 é um gás de cor castanho-avermelhada, oxidante poderoso e muito tóxico. Nas reações atmosféricas pode dar origem a O_3, HNO_3 e nitratos orgânicos que contribuem para fenómenos com elevado impacto ambiental, como chuvas ácidas e eutrofização de lagos e rios (Rogerieux et al., 2007).

Este poluente é gerado nas reações de combustão dos motores a explosão, na queima de querosene, nas combustões industriais e oxidação na atmosfera ou processos que envolvem reações com espécies reativas de oxigénio (Harris e Manning, 2010). Nas áreas urbanas, as concentrações de NO_2 são bastante elevadas, devido a maior tráfego de veículos motorizados e processos de aquecimento (Harris e Manning, 2010).

$$2NO\ (g) + O_2\ (g) \rightarrow 2NO_2\ (g)$$

O principal efeito tóxico de NO_2 no organismo humano é o aumento de problemas respiratórios. Este gás aumenta a suscetibilidade a infeções respiratórias, diminuindo a imunidade do organismo na resistência a patologias como pneumonia, tosse, gripe, bronquite e náuseas (Roger et al., 1990).

Os asmáticos, indivíduos atópicos e crianças são os mais sensíveis a efeitos tóxicos do NO_2, e quando expostos a elevadas concentrações podem sofrer irritação pulmonar, espasmos, inchaço dos tecidos no trato respiratório superior, oxigenação reduzida de tecidos, disfunções pulmonares agudas, acumulação de fluidos nos pulmões, e mudanças notáveis na função brônquica (Moshenin, 1987; Bayram et al., 2001; Kampa e Castanas, 2008).

2.3. Efeitos de CO, O_3, SO_2 e NO_2 nos grãos de pólen

O estudo de efeito de poluentes atmosféricos nos grãos do pólen é bastante fundamental para a compreensão do impacto da poluição do ar na fertilidade e nos perfis proteicos do pólen, bem como no aumento de problemas respiratórios em indivíduos atópicos.

O CO, O_3, SO_2 e NO_2 podem interagir com o pólen, modificar a parede, afetar o seu metabolismo, respiração celular e perfis proteicos. Este fato pode levar à redução

da sua fertilidade (viabilidade e germinação), conteúdo de proteínas solúveis e potenciar os seus alergénios (Emberlin, 1995). Como resultado, podem ocorrer alterações no ciclo reprodutivo das plantas superiores, de acordo com as caraterísticas genéticas e fisiológicas específicas de cada planta, bem como provocar o aumento de reatividade de IgE em indivíduos alérgicos ao pólen (Bell e Treshow, 2002; Omasa et al., 2002; Elagoz e Manning, 2005).

Os efeitos da interação entre os poluentes atmosféricos e o pólen, dependem da concentração e propriedades químicas de cada poluente, caraterísticas fisiológicas e bioquímicas específicas dos grãos de pólen, tempo de exposição do pólen ao poluente, temperatura e humidade relativa do meio (Mumford et al., 1972; Rogerieux et al., 2007).

2.3.1. Efeitos de CO, O_3, SO_2 e NO_2 na fertilidade do pólen

A nível celular, os poluentes atmosféricos podem danificar a estrutura da parede do pólen e interferir no metabolismo celular, bem como na expressão de alguns genes (Treshow e Anderson, 1989; Koch et al., 1998; Roshchina e Roshchina, 2003; Kangasjärvi et al., 2005). Contudo, a parede do pólen possui antioxidantes (*como carotenóides*) captadores de espécies reativas de oxigénio, que podem inibir a ação adversa de gases atmosféricos, prevenindo e reparando os danos oxidativos (Castillo et al., 2005). Porém, este fenómeno ainda não foi confirmado experimentalmente.

Chichiriccò e Picozzi (2007), levantaram a hipótese de que a ação oxidativa de poluentes provoca inativação de alguns fatores envolvidos na germinação do pólen, pois, as diferentes etapas do processo de germinação são sensíveis aos poluentes. De ponto de vista metabólico, a respiração celular é o processo fundamental para ocorrer a germinação do pólen (White et al., 1968; Brown e Borutaite, 2004). Assim, o CO e NO_2 têm uma afinidade para se ligar com os citocromos mitocondriais e bloquearem potencialmente a cadeia respiratória, e como consequência criam-se barreiras na respiração celular (Brown e Borutaite, 2004). O_3, NO_2 e seus derivados (superóxidos, radicais hidroxila, peróxidos e compostos azotados) provocam efeitos prejudiciais nas membranas celulares, e o O_3 pode até causar a desintegração da membrana citoplasmática e lise dos organelos (Black et al., 2000; Wheeler et al., 2001; Omasa et al., 2002). Devido a

sua forte atividade oxidativa, estes gases, afetam biomoléculas celulares, como proteínas, lípidos e ácidos nucleicos, que são o material de reserva principal do pólen, podendo interferir na germinação e no desenvolvimento do tubo polínico (Harrison e Feder, 1974; Chichiriccò, 2000; Omasa et al., 2002). Assim, pode ser provável que o dano oxidativo seja o principal responsável pela inibição de germinação dos grãos de pólen (Roshchina e Mel'nikova, 2001).

2.3.2. Efeitos de CO, O_3, SO_2 e NO_2 no conteúdo proteico e perfil de polipeptídeos do pólen.

A poluição do ar é um fator de stress, podendo afetar a expressão de proteínas integrantes no sistema de defesa da planta, como relatado para o pólen de *Cupressus arizonica* em uma área de tráfego elevado em Toledo, Espanha (Cortegano et al., 2004).

Os poluentes atmosféricos podem provocar uma diminuição do conteúdo de proteínas solúveis do pólen (Parui et al., 1998). Este fato foi constatado no pólen de *Lagerstroemia* e *Junceum Spartium* exposto a SO_2, NO_2 e CO em áreas poluídas da cidade de Teerão, onde observaram a redução do teor de proteínas solúveis no pólen exposto a poluentes quando comparado com as amostras do pólen das áreas não poluídas (Rezanejad et al., 2003; Rezanejad, 2007). O mesmo fenómeno foi observado por Bist et al., (2004) com o pólen de *Ricinus communis*, exposto a SO_2 e NO_2. Estes autores verificaram uma diminuição significativa do conteúdo proteico, quando comparado com amostras não expostas, e esta diminuição foi aumentando com o aumento de concentração de SO_2 e NO_2 e o tempo de exposição do pólen aos poluentes.

Apesar de os poluentes reduzirem o teor de proteínas solúveis, vários investigadores não relatam diferenças mensuráveis nos perfis polipeptídicos de extratos proteicos de pólen exposto e não exposto a poluentes atmosféricos. Contudo, Majd et al. (2004), observaram um desaparecimento de algumas bandas de polipeptídeos correspondentes a proteínas de 22 e 45 kDa, nas amostras do pólen de *Canna* exposto à poluição atmosférica em Teerão. Além disso, Rezanejad *et al.,* (2003) observaram uma diminuição significativa na intensidade da coloração de várias bandas de polipeptídeos de proteínas solúveis do pólen de *Lagerstroemia* exposto *in vivo* a SO_2, NO_2 e CO durante 10 e 20 dias, em zonas

poluídas da cidade de Teerão. Estes dados sugerem que a poluição do ar pode efetivamente promover modificações estruturais nas proteínas presentes no pólen (Chichiricco e Picozzi, 2007).

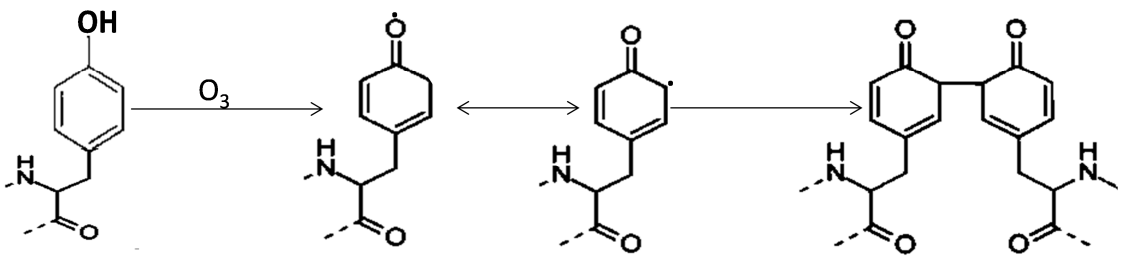
2.3.3. Efeitos de CO, O_3, SO_2 e NO_2 na alergenicidade do pólen

Alergia é uma resposta anormal e específica do sistema imunológico a uma substância estranha ao organismo, ou seja, uma hipersensibilidade imunológica a um estímulo externo específico. O anticorpo envolvido em reações alérgicas em seres humanos é o IgE. A interação específica entre o alergénio e IgE ligada ao mastócito resulta na libertação de histamina (*mediador inflamatório*) causando vasodilatação, aumento da permeabilidade vascular, contração de músculo liso e quimioatração de outras células inflamatórias. Dai que, mais da metade dos pacientes com doenças alérgicas apresentam altos níveis de IgE (Pathak e Palan, 2005).

A doença causada por reação de hipersensibilidade das vias respiratórias, induzida por grãos de pólen é denominada por polinose, sendo caraterizada por manifestações alérgicas sazonais em indivíduos sensíveis, por afetar os pacientes durante a estação polínica (Peternel et al., 2007).

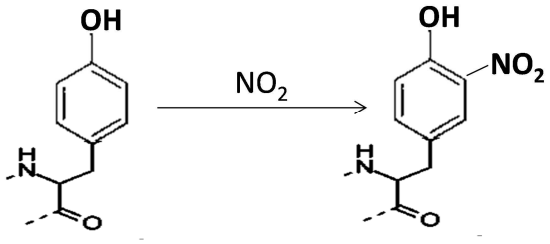
O mecanismo químico e bioquímico da interação dos poluentes do ar e os alergénios do pólen ainda não é bem compreendido, contudo, estudos revelam que as substâncias químicas atmosféricas podem induzir a alteração de proteínas de grãos de pólen e aumentar a sua digestão intracelular, podendo potenciar a sua alergenicidade. Sousa et al. (2012), revelam que SO_2 e NO_2 aumentam a alergenicidade do pólen de *Acer negundo*.

O O_3 pode modificar a estrutura química de proteínas do pólen através da geração de espécies reativas de oxigénio, o que pode potenciar os seus alergénios (Bowler e Crapo, 2002).



O O_3 pode ainda aumentar a permeabilidade da membrana das células epiteliais, facilitando a penetração de alergénios do pólen nas vias aéreas, levando assim a um aumento de libertação de mediadores inflamatórios em indivíduos atópicos (Bayram et al., 2001).

O NO_2 pode aumentar a alergenicidade do pólen, através da nitração das proteínas alergénicas (Franze et al., 2005; Gruijthuijsen et al., 2006). A nitração de proteínas afeta principalmente os resíduos de tirosina, resultando na formação de nitrotirosina. Os polipeptídeos nitrados apresentam epítopos mais ativos e aumentam a reatividade de alergénios com anticorpos IgE (Gruijthuijsen et al., 2006).



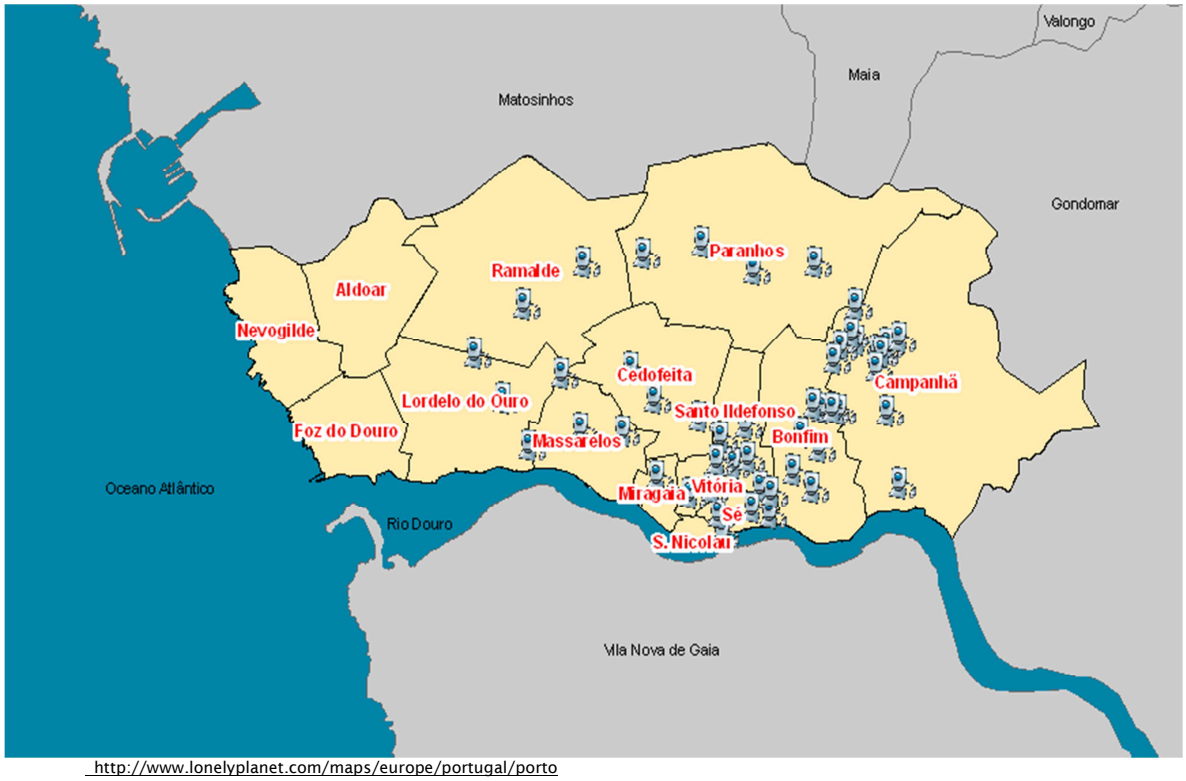
O CO e SO_2 podem distorcer a morfologia polínica e induzir o aumento de aminoácidos livres nos conteúdos proteicos de grãos do pólen, facto que pode estar associado a exacerbação de reações alérgicas em indivíduos expostos (Ruffin et al., 1986; Bist et al., 2004).

2.4. Descrição da área de estudo

A área de estudo foi a cidade do Porto, segunda maior cidade de Portugal, localizada na zona norte do país, ocupa uma área de 41,66 km^2, é limitada a oeste pelo Oceano Atlântico e ao sul pelo rio Douro *(Fig 2) (*Teixeira, 1985). Possui um clima temperado com temperaturas médias de 13 °C no inverno e cerca de 25 °C no verão.

Porto, foi tomado como área de pesquisa, devido ao aumento de prevalência de alergias respiratórias nos últimos anos, conforme os registos, nos hospitais desta cidade. Na cidade do Porto, a *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus* são usadas como plantas ornamentais no jardim Botânico e no parque da cidade. Analogamente, esta cidade possui uma densidade industrial acentuada,

tornando-se um dos maiores consumidores de energia no norte de Portugal. As principais fontes emissoras de poluentes atmosféricos são as indústrias de petróleo, petroquímicas, termoelétricas, unidades de incineração, atividades portuárias e o intenso tráfego de veículos motorizados (Sousa *et al.*, 2012).



http://www.lonelyplanet.com/maps/europe/portugal/porto

Fig 2: Mapa da cidade do Porto

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Capitulo III

Germinação *in vitro* do Pólen de Betulaceae

3. *In vitro* germination of Betulaceae pollen

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In vitro germination of Betulaceae pollen

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Abstract:	Evaluation of pollen fertility is important for various aspects related with plant productivity such as plant breeding programmes or in cultivar selection. In this work sixteen culture medium were tested for in vitro germination of Ostrya carpinifolia Scop, Carpinus betulus L., Betula pendula Roth, Alnus glutinosa L. and Corylus avellana L. pollen in order to find the most suitable one. The media tested for germination contained two different concentrations of sucrose, H3BO3 and calcium (in the form of calcium nitrate (Ca(NO3)2) or chloride (CaCl2). Al media were tested with and without L-proline. The incubation time was 24 h to 25º C. The results show that the best germination rates were obtained in the media containing L-proline. Appart from Betula pendula Roth, pollen germination and pollen tube growth increased by the addition of a source of calcium to the medium.
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In vitro germination of Betulaceae pollen

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Abstract

Evaluation of pollen fertility is important for various aspects related with plant productivity such as plant breeding programmes or in cultivar selection. In this work sixteen culture medium were tested for in vitro germination of *Ostrya carpinifolia* Scop, *Carpinus betulus* L., *Betula pendula* Roth, *Alnus glutinosa* L. and *Corylus avellana* L. pollen in order to find the most suitable one. The media tested for germination contained two different concentrations of sucrose, H_3BO_3 and calcium (in the form of calcium nitrate ($Ca(NO_3)_2$) or chloride ($CaCl_2$). Al media were tested with and without L-proline. The incubation time was 24 h at 25º C. The results show that the best germination rates were obtained in the media containing L-proline. Appart from *Betula pendula* Roth, pollen germination and pollen tube growth increased by the addition of a source of calcium to the medium.

Keywords: Betulaceae; pollen, in vitro germination; L-Proline.

Introduction

Germination leads to the formation and growth of pollen tube that carries the male gametes to the embryo sac in order to occur the double fertilization in flowering plants. The methodologies usually used to evaluate pollen fertility can be diverse such as staining techniques, germination or seed set percentage (Dafni and Firmage, 2000; Heslop-Harrison et al., 1984; Stanley and Linskens, 1974).

The stigma possesses the optimal medium for pollen germination to occur, which comprises several stages of development of the pollen tube and the time taken for each stage varies greatly from species to species depending on the type of reserve material present in pollen as well as external factors (Feijo et al., 2001). Generally, for in vitro germination to occur it is necessary to prepare, under laboratory conditions, a medium with similar composition to the stigma for the pollen germinate. Usually, in vitro pollen germination requires hydration, a source of carbohydrates and boron. Additionally, the germination medium can also include other components such as calcium,

magnesium and potassium (Brewbaker and Kwack, 1963; Fan et al., 2001). All these elements can be combined at different concentrations according to plant species tested (Feijo et al., 1995; Linskens, 1964). Pollen hydration is required once, at the time of dehiscence, the pollen is released into the atmosphere dehydrated, suffering rehydration on the stigma (Wilsen and Hepler, 2007). Sucrose is normally used as the source of carbon necessary for energy supply and carbohydrate skeleton formation. Boron plays an important role in pollen germination and tube growth by the construction of the wall during the pollinic tube elongation (Feijo et al., 1995; Obermeyer and Blatt, 1995; Taylor and Hepler, 1997). Some studies point out for a specific role of calcium in the regulation of polarized growth of pollen tubes (Hepler, 2005; Michard et al., 2011). This substance may be added to the germination medium in the form of chloride or nitrate ($CaCl_2$ and $Ca(NO_3)_2$). Furthermore, incubation temperature and time are also important parameters to control (Fu et al., 2001; Kelen and Demirtas, 2003).

Several studies have been developed along the years in order to determine qualitatively and quantitatively the best germination medium for a tested species (Heslop-Harrison et al., 1984; Steer, 1989). These studies provide the basis for establishing of germination medium for species not tested in the literature.

The pollen germination assessment is important in the study of various aspects related with plant productivity such as pollen vigour during storage conditions for controlled pollination, for the choice of pollinizers, in the evaluation of cultivar compatibility, in plant breeding programmes or in cultivar selection (Cheung et al., 2010; Dafni and Firmage 2000). Pollen germination in several fruit trees, are closely related to a greater or lesser ability to produce fruit and consequently higher or lower yields (Shukla et al., 1998). The trees of the Betulaceae family comprise several genus that are usually used for timber and fruit production and also have been spread out for ornamental purposes (Chen et al., 1999). They are anemophilous trees, producing great amounts of pollen and so, have been recorded in aerobiological and allergenic studies. Furthermore, in the literature, information on in vitro germination media for all these species is scarse and the existing studies describe different methodologies and different germination media, making it difficult to compare the obtained results.

In the present work an attempt was made to compare the pollen germination capacity of five cultivated species belonging to family Betulaceae viz. *Ostrya carpinifolia* Scop., *Carpinus betulus* L., *Betula pendula* Roth., *Alnus glutinosa* L. and *Corylus avellana* L..

Material and methods

Anthers of *Ostrya carpinifolia* Scop., *Carpinus betulus* L., *Betula pendula* Roth., *Alnus glutinosa* L. and *Corylus avellana* L. were collected during the flowering season (February and March) in the botanic garden of Porto, Portugal. They were dried at 27°C, gently crushed and the pollen thus released was passed through different grades of sieves to obtain pure pollen. Pollen samples were then stored at –20°C.

Pollen germination was assayed in eight different germination medium at two sucrose concentration levels (15% and 25%). All medium contained boric acid (H_3BO_3), but several have also Ca in form the nitrate ($Ca(NO_3)_2$) or chloride($CaCl_2$). Comparison between different concentrations of boron and calcium were tested (a total of 16 different combinations) (Table 1). These elements were selected based on reference literature available.

Pollen grains were distributed in petri dishes with medium and maintained in a thermocontrolled dryer at 25 °C in the dark. The germination was scored after 24 hours of incubation and pollen was considered germinated when the tube has at least twice the diameter of pollen. In order to calculate the germination percentage, two replications per plant were performed and in each one five fields per sample (each one containing 100 pollen grains) were counted using a light microscope (Leica DM LB).

Results

In this work, the comparison of the pollen germination capacity of five species of trees belonging to the Betulaceae family, namely *Ostrya carpinifolia* Scop, *Carpinus betulus* L., *Betula pendula* Roth, *Alnus glutinosa* L. and *Corylus avellana* L. was performed. A total of 16 solid media were used and tested with and without L-proline. The greater values of the germination were obtained with *Ostrya carpinifolia* pollen around 43.3% in a medium containing 200 ppm H_3BO_3, 200 ppm

$CaCl_2$, 400 ppm L-proline and 25% sucrose; *Carpinus betulus* pollen around 36% in the medium with 200 ppm H_3BO_3, 400 ppm $Ca(NO_3)_2$, 400 ppm de L-proline and 15% sucrose; *Betula pendula* pollen with 39.9% in the medium containing 200 ppm H_3BO_3, 400 ppm L-proline and 15% sucrose. *Alnus glutinosa* and *Corylus avellana* pollen had germination of 31.6% and 28.4% respectively in a medium with 100 ppm H_3BO_3, 100 ppm $CaCl_2$, 400 ppm L-proline and 15% sucrose (Table 1).

Discussion

In our study, the media presenting higher germination percentages for each of the five Betulaceae studied contained L-proline. Some studies showed that levels of proline in the pollen are very high, being the highest among floral organs (Chiang and Dandekar, 1995; Krogaard and Andersen, 1983; Lansac et al., 1996; Schwacke et al., 1999). Nevertheless, the reason for this accumulation has been associated with multiple functions such as free radical scavenger, protector of membranes and cellular structures, energy source or as metabolic precursor for pollen tube elongation (revised in Mattioli et al., 2012). This amino acid is therefore an important component for the successful male fertility (Mattioli et al., 2012; Zhang and Croes, 1983).

In our study, all Betulaceae species tested required boron to germinate although in different concentration. Boric acid is known to be crucial for pollen germination and tube growth. Boron deficiency can lead to a reduce in pollen germination rate, retardation of pollen tube growth, pollen tube anomalies such as the swelling at the tip of the pollen tube or tube bursting (Acar et al., 2010; Holdaway-Clarke et al., 2003; Wang et al., 2003; Yang et al., 1999).

It is reported a concentration of 100 ppm to be required for successful pollen germination for most species (Brewbaker and Majumder, 1961), whereas higher concentrations can inhibited pollen grain germination and pollen tube elongation (Potts and Marsden-Smedley, 1989; Wang et al., 2003). However in our study only *Alnus glutinosa* L. and *Corylus avellana* L. pollen germination was favored in the presence of this level of boric acid while the pollen germination of the other three species was best in the presence of higher boric acid concentration (200 ppm). Yet, the best germination medium for *Ostrya carpinifolia* Scop, *Carpinus betulus* L., *Betula pendula* Roth,

differed in the other constituents, the presence of calcium for the first two species and only the presence of boron in the last one.

Our results showed that the need of calcium for successful germination varied between the tested species. Also, when necessary, the form of how calcium is added to the germination medium and its concentration varied between the species. *Betula pendula* Roth. was the only species where pollen germination percentage was higher in the absence of calcium. This is in accordance with the results of Käpylä (1991) and Pasonen and M. Kapyla (1998) that working with *Betula pendula* pollen, reported germination percentages over 50% using a medium containing only 100 ppm of boric acid and 0.5 M sucrose. Also, these authors showed that an incubation medium containing also $Ca(NO_3)_2$ did not increased the pollen germination. Nonetheless, it has been admitted that the calcium is an important element in in the growth medium of several plant species (Brewbaker and Kwack, 1963) known to stimulate the growth of pollen tube (Hepler, 2005; Michard et al., 2011) and required for maintenance of membrane integrity (Kell and Donath, 1990; Van Stkveninck, 1965). Also in *Arabidopsis thaliana* pollen was observed that the absence of calcium did not inhibited pollen germination, however its presence improved germination percentage (Daher et al., 2009). These authors suggest that this may be related to the presence in or on the surface of the pollen of a stock of calcium.

In any pollen germination media a source of energy is a key element for successful germination since pollen tube does not perform photosynthesis and therefore needs a carbon source, usually in the form of sucrose, for energy supply and Carbohydrate skeleton formation (Daher et al., 2009). In the literature the optimal sucrose percentage varies from species to species. A study performed in *Betula luminifera* H.J.P.Winkl. pollen was observed that excess of sucrose inhibit their germination, being 10% sucrose the optimal percentage (Bao et al., 2009). The same was observed in *Corylus heterophylla* Farnch. × *C. avellana* L. where was shown that different concentrations of sucrose had significant impact on pollen germination being the optimal concentration 15% (Zhai et al., 2009). In our work only *Ostrya carpinifolia* Scop. germinated best in higher values of sucrose, 25%, the other Betulaceae species achieved higher pollen germination percentages in a medium containing 15% of sucrose.

To sum up, in our study was observed that the five Betulaceae species tested differed in their pattern of response to boric acid, calcium and sucrose. Sucrose and boric acid were essential for pollen germination while the need for calcium and its form was species dependent. In terms of the optimal level of boric acid *Alnus glutinosa* L. and *Corylus avellana* L. pollen required less concentration than the other species. Appart from *Betula pendula* Roth. pollen calcium improved the germination percentage.

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Table 1 – Pollen germination results

Germination medium																	
	H3BO3 (ppm)			200				200				100				200			
	CaCl2 (ppm)			x				x				100				200			
	Ca(NO3)2 (ppm)			400				x				x				x			
	L-proline (ppm)	x	x	400	400	x	x	400	400	x	x	400	400	x	x	400	400
	Sacarose (%)	15	25	15	25	15	25	15	25	15	25	15	25	15	25	15	25
percentage	*Betula pendula*	3.6	1.0	1.6	1.2	15.0	ng	***39.9***	4.0	9.0	3.2	6.8	4.8	2.0	ng	4.4	1.6
	Alnus glutinosa	2.4	4.8	6.4	4.8	ng	12.8	10.0	11.4	23.2	9.8	***31.6***	9.0	24.6	10.0	28.8	12.6
	Corylus avellana	8.0	ng	4.6	4.2	ng	ng	19.0	3.0	25.6	ng	***28.4***	6.0	8.8	4.6	4.6	3.6
	Ostrya carpinifolia	10.2	4.0	2.8	5.8	2.0	10.0	ng	5.8	8.6	12.6	10.0	13.4	10.0	19.6	6.2	***43.3***
	Carpinus betulus	15.8	ng	***36.0***	11.6	ng	2.6	ng	2.4	3.6	4.2	2.4	8.2	9.8	7.0	5.6	9.2

Capitulo IV

Efeitos de CO, O_3 e SO_2 na Viabilidade, Germinação, Conteúdo Proteico e Perfil de Polipeptídeos do Pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus*

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

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Keywords:	Betula pollen, Atmospheric pollutants, Pollen viability, Pollen germination, Protein content

Exposure of *Betula pendula* Roth pollen to atmospheric pollutants CO, O_3 and SO_2

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ABSTRACT

Betula pendula Roth pollen, under laboratory conditions, was exposed to three atmospheric pollutants: CO, O_3 and SO_2. Two levels of each pollutant were used and the first level corresponds to a concentration on the atmospheric hour-limit value acceptable for human health protection in Europe and the second level to a higher concentration level (about the double). Experiments were done under artificial solar light with temperature and relative humidity controlled. Our results indicated that in urban areas, concentrations of CO, O_3 and SO_2 on the limits established for human protection can affect the pollen fertility because 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.

1. Introduction

Pollen is a natural atmospheric environmental allergenic which is responsible for several more or less severe human health effects (Linskens and 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 that are emitted from anthropogenic sources such as carbon monoxide (CO) resulting from incomplete combustion of carbon materials, ozone (O_3) which is a secondary pollutant generated in photochemical smog and sulfur dioxide (SO_2) emitted in the 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 sensitization (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 the Spermatophytes (Bell and Treshow, 2002; Omasa et al., 2002; Elagoz and Manning, 2005). At cellular level air pollutants provoke the damage of the membrane structures and interfere with cellular mechanisms as well as in the gene expression (Koch et al., 1998; Roshchina and Roshchina, 2003; Kangasjärvi et al., 2005). There are some evidence, but not confirmed experimentally, that the pollen wall contains antioxidant species, like the carotenoids, that trap reactive oxygen species and reduce the negative role of air pollutants (Castillo et al., 2005).

The adverse effects of air pollutants on the fertility are usually detected during the pollen germination (Feder and 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 presents in the pollen. In terms of metabolism, the respiration is the fundamental factor for the germination due energy produced (Brown and Borutaite, 2004; White et al., 1968). The air pollutant CO has affinity towards the mitochondria cytochromes being potential inhibitor of the cellular respiration (Brown and Borutaite, 2004). NO_2,

O_3 and its reactive species derivatives (superoxides, hydroxyl radicals, peroxides and nitrogen containing reactive species) provoke damage of the cellular membrane and O_3 may even cause cellular organelle disconnection (Black et al., 2000; Omasa et al., 2002; Wheeler et al., 2001). At molecular level, air pollutants have a relatively strong oxidative role that affects biomolecules like proteins, lipids and nucleic acids that constitute the main material pollen reservoir, interfering with pollen germination and elongation of the pollinic tube (Chichiriccò, 2000; Harrison and Feder, 1974; Omasa et al., 2002, Roshchina and Mel'nikova, 2001).

Betula tree, also known as birch, is a genus of the Betulaceae family and due its easy-growing has been spread out for ornamental purposes on the parks and gardens in the urban area of Porto. They are anemophilous trees, producing great amounts of pollen and so, have been recorded in aerobiological and allergenic studies. Their pollen is one common source of pollinosis in Europe (D'Amato et al, 2007). However, sensitization to pollen allergens is variable between different regions of the world, according to climate, urbanization levels, geography, and vegetation (Du Bay and Murdy, 1983).

The aim of this research was to study the effects of CO, O_3 and SO_2 on viability, germination and protein content of *Betula pendula* Roth pollen. This objective was achieved by exposing, under controlled experimental conditions, pollen samples at two different gas concentration values: just below the safety standard limits for human health protection and to an higher level corresponding roughly to about the double of the first levels during one and two days.

2. Material and methods

2.1. Pollen samples

During the flowering season, male catkins of the *Betula pendula* Roth were collected from Botanic garden and City Park of Porto and dried at 27ºC. After 2 days were gently crushed and

passed through different grades of sieves to obtain pure pollen. This pollen was stored at -20ºC until use.

2.2. In vitro exposure to pollutants

Pollen samples were artificially exposed in a environmental chamber, made of acrylic and wood mix with dimensions about 50 x 70 x 50 cm. This chamber was built and tested by our team (Sousa et al, 2012). In this chamber, sunlight was simulated by a Solar Simulator (Newport Oriel 96000 150 W) with Air Mass Filters 1.5 Global 81094 and a liquid light guide positioned 15 cm above the sample holder. A fan (SUNON SF23080AF) was used to homogenize the air inside the chamber. Air temperature and relative humidity were continually monitored using an EBRO EBI20 sensor. The gas concentrations were monitored throughout each assay by AEROQUAL Series 500 sensors, registering data every minute. These sensors were connected to an external computer.

Each pollen sample [300 mg of dry weight (DW)] was exposed individually with CO, O_3, and SO_2, under two different concentrations: a first concentration level corresponding to the current atmospheric hour-limit value acceptable for human health protection in Europe (8.73 ppm for CO, 0.061 ppm for O_3 and 0.13 ppm for SO_2) (European Union Directive 2008/50/EC of 21 May 2008 on ambient air quality and cleaner air for Europe) during 6h assay; and, a second level corresponding to about the double of the first concentration level. SO_2 (99.9% v/v) concentrations were attained by injecting gas directly obtained from bottles of compressed gas (Sigma-Aldrich). CO was generated by chemical reaction of sulfuric acid with formic acid– it was generated by mixing, in a test tube, stoichiometric amounts of the two concentrated acids and subsequently injected into the chamber. Three injections of gas per assay were performed using a micro syringe within each 2h interval in order to prevent greater concentration fluctuations. Ozone was generated using an A2Z OZONE system coupled to an OMRON H3DK-S1 timer, controlling the injection of ozone for brief periods of time in order to offset the high ozone reactivity. Non-exposed pollen samples were used as control.

Table 1 shows the exposure experiments that were performed and the corresponding pollutants levels and environmental conditions.

2.3. Pollen viability

Pollen viability was estimated using the trypan blue stain, which is a vital dye and its reactivity is based on the fact does not interact with the cell unless the membrane is damaged. So, all the cells non-colored are viable. Pollen grains (2 mg) are placed for 5 min in 0.5 ml of 60% trypan blue and centrifugation for 3 min at 13 200 rpm. Then 20 µl were placed on a microscope slide and observed at light microscope (DMLB: Leica). Pollen viability was determined as the percentage of non-colored 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.

2.4. In vitro pollen germination

Non-exposed and exposed pollen samples were germinated at 27°C in the dark for 24 h in a germination medium optimized by the authors (200 ppm H_3BO_3, 400 ppm L-proline, 15% sucrose, 0.5% agar). Two replicates were performed and in each one, five random fields were counted (100 pollen grains per field) using a light microscope (DMLB: Leica). A pollen grain was classified as germinated when the tube was longer than the pollen size (Nepi and Franchi, 2000).

2.5. 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 4 h. The suspension was centrifuged at 13200 rpm for 30 min at 4 °C. The supernatant was filtered through a 0.45 µm Millipore filter and centrifuged once again. The soluble protein content of all pollen

extracts was quantified colorimetrically with the Coomassie Protein Assay Reagent (Pierce) by the Bradford Method (Bradford, 1976).

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

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

3. Results and discussion

3.1. Pollen viability

The results of *Betula pendula* Roth pollen viability before and after exposition to the three pollutants are showed in the Table 2. A global analysis of this table shows that the percentage of viable pollen decreases whatever the pollutant used, namely non exposed pollen shows a percentage of viability of 93±1% while the pollen exposed to the three gases, at the highest concentration, shows a percentage of viability of about 80%. An important result is that, even at the lowest pollutant level studied, which is below the safety standard limit for human health, a decrease on the viable pollen was observed.

The analysis of variance (ANOVA) of the effect of the pollutant exposition on the pollen viability (Table 3) shows that it is statistically significant.

3.2. Pollen in vitro germination rates

The results of the in vitro germination rates of *Betula pendula* Roth pollen before and after exposition to the three pollutants are also shown in Table 2. A global analysis of these results shows that the percentage decreases for the three pollutants under analysis, namely non exposed

pollen shows a percentage of germination of 39±1% while the exposed pollen, at the highest concentration, shows germination percentages about 25%. These results agree with our viability results previously discussed. The analysis of variance (ANOVA) of the effect of the pollutant exposition on the pollen germination (Table 3) shows that it is statistically significant.

The analysis of the germination results for the three pollutants shows that the type of chemical substance has a similar effect on the germination of the pollen – about 25% at the highest pollutant level. Also, and confirming the viability results, the in vitro germination of the pollen was also affected even for the lowest pollutant level studied – about 29% at the lowest pollutant level.

Our results are similar with those obtained for the *Crocus vernus* (*Iridaceae*) (Chichiriccò and Picozzi, 2007) that observed a 50% germination rate when pollen was independently exposed to 0.5 ppm CO and 0.3 ppm O_3 and lower germination rates when the concentrations increased to 25 ppm CO and 0.5 ppm O_3. In another in vitro study, a 40 to 50% germination rate decrease was observed for *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 exposed to 0.6 ppm of SO_2 for three and eight hours during the flowering season (Du Bay and 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 pollinic tube was observed.

The results obtained in this work, showed that even under limiting CO, O_3 and SO_2 concentrations considered safe for the protection of human health, the viability and in vitro germination of *Betula pendula* Roth pollen were affected. However, the effect of the three pollutants was similar probably because relatively small concentrations were used in the pollen exposition 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).

3.4. Protein pollen content

The protein content of the *Betula pendula Roth* pollen before and after exposure to pollutant gases is shown in Table 2. The analysis of the results shows a general decreasing trend in protein content of the exposed samples when compared with the control. However, the one-way analysis of variance of this effect shows that it is not significant for a 0.05 probability level with the exception of SO_2 ($F = 6.2 > F_{crit}$). Indeed, SO_2 was the pollutant that induced a consistent decrease on the protein content (750±28, 695±28 and 726±24 µg/mL) when compared with the control (1238±355 µg/mL). The exposure with the other two pollutants provoked only a small decrease in the pollen soluble protein content when compared with the control.

Accordingly 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 defense system as reported for the *Cupressus arizonica* pollen in a high traffic area in Toledo, Spain (Cortegano et al., 2004). Similar results were observed for *Lagerstroemia indica* and *Spartium junceum* L pollen exposed to SO_2 and CO in polluted areas of Teheran (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 SO_2 showed that the total soluble protein content decreased as 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).

Although, as discussed above, the protein content of the exposed pollen showed some variation relatively to the non exposed pollen, no statistically reliable changes in the polypeptide profile between exposed and non-exposed pollen samples were detected (Fig.2). The electrophoretic profile revealed several polypeptide bands ranging from 70 to 15 kDa after Coomassie staining of the gel. The polypeptide profiles of all the pollen samples showed five clusters of bands about of 70, 55, 35, 25 and 15 kDa.

Other studies report the existence of no measurable differences in the polypeptide profiles of pollen extracts exposed and no exposed to atmospheric pollutants (Rezanejad, 2007). The disappearance and/or intensity reduction of certain bands corresponding to soluble proteins of 22 and 45 kDa, in the protein profile of *Canna indica* pollen exposed to the Teharan atmospheric pollution was referred by Majd et al. (2004). Also, Rezanejad et al. (2003) observed a significant decrease in the coloration intensity of several bands corresponding to soluble proteins when *Lagerstroemia* pollen was exposed to SO_2 and CO for 10 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 and Picozzi, 2007).

The effect of air pollutants on the polypeptide profile of pollen is a subject that deserves further research. The results obtained in the present study are inconclusive, probably, because the time of exposure of the pollen to the three pollutants was relatively small. Further studies should focus on the concentration of the pollutants and exposure time in order to obtain information about the cellular mechanisms that are affected and about the biochemical modifications on the proteins.

4. Conclusions

Betula pendula Roth pollen when exposed to the air pollutants CO, O_3 and SO_2 at levels that can be considered safe for human health protection show significant negative effects on its fertility namely on its viability and germination potential. Also, an apparent effect on the protein content of the exposed samples when compared with the control was detected but it lacks a clear statistical significance.

Taking into consideration that these results were obtained at concentration close to the limiting values for the protection of human health it 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.

Acknowledgments

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Table 1 – Average and standard deviation of the concentration of CO, O_3, SO_2 and NO_2, temperature and relative humidity during the *Betula* pollen exposure experiments (+, 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).

Pollutant	Experiment	Concentration (ppm)	Temperature (ºC)	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.

Table 2 – Results of the viability, germination and total soluble protein (TSP) content of *Betula* pollen exposed to CO, O_3, SO_2 and NO_2. Signs represent gas concentration level, namely: +, about the hour-limit value; ++1d, about two times hour-limit value during one day; ++2d, about two times hour-limit value during two days. *

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.

Table 3 - Effect of the *in vitro* exposure to pollutant gases (CO, O_3 and SO_2) of *Betula pendula* Roth 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

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

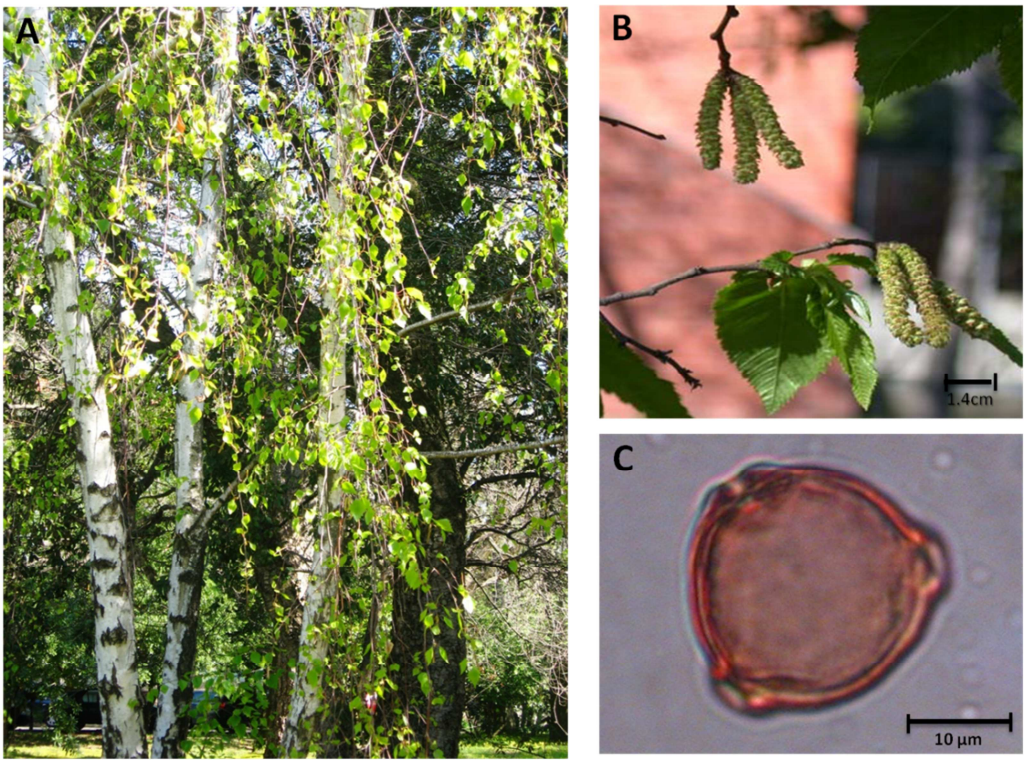

Fig. 1 – *Betula* trees (A), staminate flowers (B) and the corresponding pollen observed by optical microscope (C).

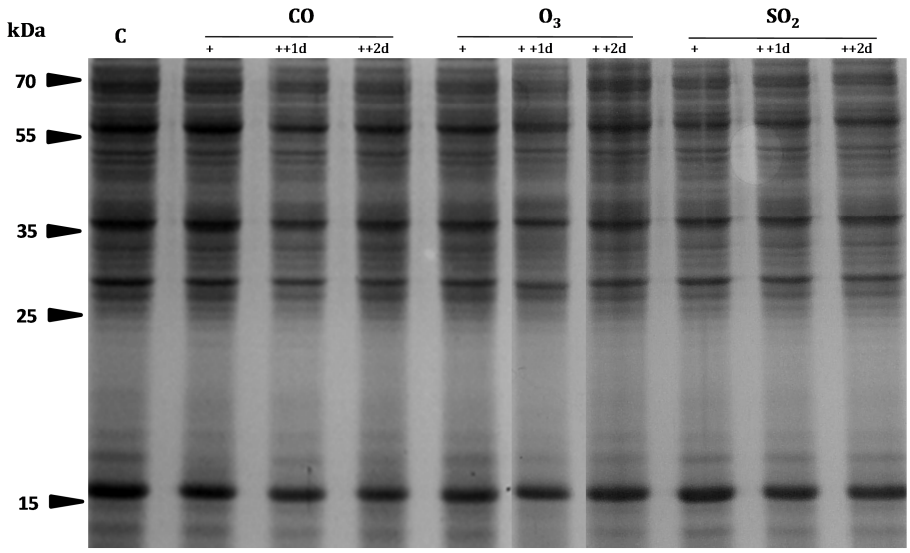

Fig. 2 – SDS-PAGE of soluble protein of *Betula* pollen extracts exposed to CO, O_3 and SO_2. Signs above the lanes represent gas concentration level. C – control; +, about the hour-limit value; ++1d, about two times the hour-limit value during one day ; ++2d, about two times the hour-limit value during two days.

4.2 *In vitro* exposure of *Ostrya carpinifolia* and *Carpinus betulus* pollen to atmospheric levels of CO, O_3 and SO_2

Environmental Science and Pollution Research

In vitro exposure of Ostrya carpinifolia and Carpinus betulus pollen to atmospheric levels of CO, O3 and SO2

--Manuscript Draft--

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Abstract:	Ostrya spp. and Carpinus spp. pollen were exposed in vitro to three atmospheric pollutants: CO, O3 and SO2. Two levels of each pollutant were used and the first level corresponds to a concentration about the atmospheric hour-limit value acceptable for human health protection in Europe and the second level to about the double of the first level. Experiments were done under artificial solar light with temperature and relative humidity controlled. The percentage viability of the exposed pollen samples showed a significant decrease. Also, the germination percentage showed a significant decrease in both exposed pollen and the effect was most pronounced for SO2, followed by O3 and CO. A general decreasing trend in the total soluble protein content of the exposed pollen samples when compared with the control was observed but it was only statistically significant for the Ostrya spp pollen. The results showed that marked effects were observed on the Ostrya spp. and Carpinus spp. pollen when exposed to air pollutant levels that can be considered safe for human health protection.
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In vitro exposure of *Ostrya carpinifolia* and *Carpinus betulus* pollen to atmospheric levels of CO, O_3 and SO_2

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Abstract

Ostrya spp. *and Carpinus* spp. *pollen* were exposed *in vitro* to three atmospheric pollutants: CO, O_3 and SO_2. Two levels of each pollutant were used and the first level corresponds to a concentration about the atmospheric hour-limit value acceptable for human health protection in Europe and the second level to about the double of the first level. Experiments were done under artificial solar light with temperature and relative humidity controlled. The percentage viability of the exposed pollen samples showed a significant decrease. Also, the germination percentage showed a significant decrease in both exposed pollen and the effect was most pronounced for SO_2, followed by O_3 and CO. A general decreasing trend in the total soluble protein content of the exposed pollen samples when compared with the control was observed but it was only statistically significant for the *Ostrya* spp pollen. The results showed that marked effects were observed on the *Ostrya* spp. *and Carpinus* spp. pollen when exposed to air pollutant levels that can be considered safe for human health protection.

Keywords: *Ostrya* pollen; *Carpinus* pollen; atmospheric pollution; viability and germination; soluble protein profile.

Introduction

Pollen is considered as an atmospheric biopollutant that is correlated with respiratory allergies in humans (Bosch-Cano et al. 2011; D'Amato et al. 2010, Linskens and Cresti 2000; Sousa et al. 2011, Traidl-Hoffmann et al. 2009). Besides pollen there are also significant associations between regulated air pollutants exposition and allergy related diseases in humans (Kim et al. 2013).

The study of the effect of atmospheric pollutants on the pollen allergenicity is currently a scientific relevant subject (Sousa et al., 2012). Regulated air pollutants, such as carbon monoxide (CO), ozone (O_3) and sulphur dioxide (SO_2), may have a synergistic effect when pollen is present. Indeed, both air pollutants and pollen have a negative impact on human health and, when both are present, an increase risk may exist, but this is not regulated. Nevertheless, the effect of air pollutants in the allergenic potential of pollen is not completed understood but it has been suggested that they increase the allergenicity by increasing the pollen antigens (Bosch-Cano et al. 2011; Sousa et al. 2012; Traidl-Hoffmann et al. 2009).

Atmospheric pollutants interact with pollen provoking modifications on their protein content and on its fertility resulting in alteration of the reproductive cycling of spermatophytes (Bell and Treshow 2002; Elagoz and Manning 2005; Omasa et al. 2002.). At cellular level air pollutants induce modifications on the membrane structures, interfere in the cellular mechanisms and in gene expression processes (Kangasjärvi et al. 2005; Koch et al. 1998; Roshchina and Roshchina 2003; Treshow and Anderson 1989). There some evidences that the wall of pollen contain antioxidant species (like the carotenoids) that react with reactive oxygen species with potential to inhibit the negative effects of atmospheric gases (Castillo et al. 2005).

The adverse effects of air pollutants on pollen occur during its germination (Feder and Sullivan 1969). Taking this information into consideration it was hypothesized that air pollutants would inactivate by oxidation some factors involved in pollen germination (Chichiricco and Picozzi 2007). From the metabolic point of view the crucial factor for germination is the cellular respiration beginning in the cytosol and ending in the mitochondria (Brown and Borutaite 2004; White et al. 1968). CO has affinity towards mitochondrial cytochrome and upon binding can stop the respiratory chain (Brown and Borutaite 2004). O_3 and its reactive derivatives (superoxides,

hydroxide radicals, peroxides and nitrogen reactive compounds) provoke deleterious effects on cellular membranes and O_3 can cause the disconnection of the organelle (Black et al. 2000; Omasa et al. 2002; Wheeler et al. 2001). At molecular level, these gases show a high oxidative potential that can damage biomolecules present in the cellular compartments, such as proteins, lipids and nucleic acids, interfering in the development of the pollinic tube (Chichiriccò 2000; Harrison and Feder 1974; Omasa et al. 2002; Roshchina and Mel'nikova 2001).

In this paper the results obtain by in vitro fumigation of *Ostrya* spp. and *Carpinus* spp. pollen with three air pollutants (CO, O_3 and SO_2) will be presented and discussed. The pollen of these two plants was chosen because it is usually associated with respiratory allergies in Europe (Filon et al. 2000; Çeter et al. 2012; Gumowski et al. 2000). In this study the pollen of the two plants were exposed at two levels of concentration for each pollutant: the lower level corresponds to about the limiting value recommended in the European Union for human health protection; the higher level corresponds to about the triple of the lower level. The lower concentration was used in one day exposition (a period of six hours with artificial sun light) and the higher concentration was used for one and two days (a total period of thirty hours exposition: 12 hours with artificial sun light and eighteen hours in the dark). The objective of this research is to assess the effect of relatively lower concentration and shorter expositions of the three previous air pollutants in the germination, viability and soluble protein profiles of the pollen of the two plants.

Materials and methods

Pollen samples

Ostrya and *Carpinus* are plants used in the ornamental of gardens and parks in urban centres. They are trees of medium to large deciduous foliage (Fig. 1.A). They produce staminate flowers and the male form in clumps strobiloides pending and terminal (Fig 1.B) (Chen et al., 1999). The anthers of the two trees were collected during the flowering season (first days of spring) from the Botanic Garden and City Park of Porto and were dried at 27°C, gently crushed and the

pollen thus released was passed through sieves of different grades in order to obtain pure pollen (Fig. 1.C). Pollen samples were stored at –20 °C.

Fumigation chamber system

Pollen samples were *in vitro* exposed in a chamber made of acrylic and wood with dimensions about 50 x 70 x 50 cm and previously described (Sousa et al. 2012). In this chamber, sunlight was simulated by a Solar Simulator (Newport Oriel 96000 150 W) with Air Mass Filters 1.5 Global 81094 and a liquid light guide positioned 15 cm above the sample holder. A fan (SUNON SF23080AF) was used to homogenize the air inside the chamber. Air temperature and relative humidity were continually monitored using an EBRO EBI20 sensor. The three gases concentration was 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, O_3, and SO_2, under two different concentrations: a first level corresponding to the current atmospheric hour-limit value acceptable for human health protection in Europe (8.73 ppm for CO, 0.061 ppm for O_3 and 0.13 ppm for SO_2) (European Union Directive 2008/50/EC of 21 May 2008 on ambient air quality and cleaner air for Europe) during 6 hours assay (+1d); and, a second level corresponding to about the double of the first concentration level, during 6 hours (++1d) and two days (++2d). SO_2 (99.9% v/v) concentrations were attained by injecting gas directly obtained from bottles of compressed gas (Sigma-Aldrich). CO was generated by chemical reaction of sulfuric acid with formic acid – it was generated by mixing, in a test tube, stoichiometric amounts of the two concentrated acids and subsequently injected into the chamber. Three injections of gas per assay were performed using a micro syringe within each 2h interval in order to prevent greater concentration fluctuations. Ozone was generated using an A2Z OZONE system coupled to an OMRON H3DK-S1 timer, controlling the injection of ozone for brief periods of time in order to offset the high ozone reactivity. Pollen samples were placed into a Falcon tube with the bottom cut off and 23 µm grids (SEFAR PET 1000) were placed on both sides – this sampling system allows the circulation of the air through it and the re-suspension of the pollen. Non-exposed pollen

samples were used as control. Table 1 shows the characteristics of the *in vitro* experiments that were performed.

Pollen characterization

Pollen viability was estimated using the trypan blue stain, which is a vital dye and its reactivity is based on the fact does not interact with the cell unless the membrane is damaged. So, all the non-colored cells are viable. Pollen grains (2 mg) are placed for 5 min in 0.5 ml of 60% trypan blue and centrifugation for 3 min at 13 200 rpm. Then 20 µl were placed on a microscope slide and observed at light microscope (DMLB: Leica). Pollen viability was determined as the percentage of non-colored 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.

Non-exposed and exposed pollen samples were germinated at 27°C in the dark for 24h in the germination medium optimized by the authors: Ostrya - 200 ppm H_3BO_3, 200 ppm $CaCl_2$, 400 ppm L-proline, 25% sucrose and 0.5% agar; Carpinus - 200 ppm H_3BO_3, 400 ppm $Ca(NO_3)_2 \cdot 4H_2O$, 400 ppm L-proline, 20% sucrose and 0.5% agar. Two replications were performed for each sample and five random fields were counted (100 pollen grains per field) using a light microscope (Leica DMLB). A pollen grain was classified as germinated when the pollinic tube was longer than the pollen size (Nepi and Franchi 2000).

For the total soluble proteins (TSP) determination pollen grains (50 mg) were suspended in 1:20 (w/v) phosphate buffer saline at pH 7.4 at 4 °C. TSP were extracted in the same buffer by continuous stirring for 4 h followed by centrifugation at 13200 rpm for 30 min at 4 °C. The supernatant was filtered through a 0.45 µm Millipore filter and centrifuged one more time and TSP quantified colorimetrically with the Coomassie Protein Assay Reagent (Pierce) by the Bradford Method (Bradford 1976).

Results and discussion

Pollen viability and germination

Table 2 shows the viability percentages of *Ostrya* and *Carpinus* pollen before and after exposition to the three air pollutants. A global analysis of this table shows that the viability of both pollen samples decreased after exposition to the three gases. Table 3 shows the ANOVA results of the effect of the exposition on pollen viability and it confirms that the effect is significant for both pollen samples because for the three gases the calculated F value is always higher than the F_{crit}.

For the two pollen samples under investigation the decrease in its viability upon exposition is somewhat independent of the pollutant under consideration. Indeed, a decrease of about 10% on the viability is observed for both samples and for the highest concentration level and exposition time. Also, a significant decrease on the pollen viability is observed for the lowest concentration level and exposition time which raises some concerned because this condition corresponds to a level considered safe for human health.

Germination rates of exposed and non-exposed *Ostrya* and *Carpinus* pollen are shown in Table 2 and the ANOVA results of the air pollutant exposition effect are shown in Table 3. A global analysis of these tables show that the pollen germination significantly decreases when the pollen of both plants are exposed to the three air pollutants. Considering the highest gas concentration of each gas and highest exposition time the germination decreasing is (comparing with the non-exposed pollen germination): *Ostrya*, 43±2% to 36±1% (CO), 30±4% (O_3) and 22±1% (SO_2); *Carpinus*, 36±1% to 30±2% (CO), 23±1% (O_3) and 20±1% (SO_2). These results support the previous discussion about the pollen viability upon air pollutant exposition.

The comparison of the germination rates (and corresponding ANOVA F values, Table 3) of both pollen exposed to the three pollutants shows that the type of the chemical substance affects the extent of germination reduction. Indeed, the following decreasing trend is observed for both pollen samples: $CO < O_3 < SO_2$.

The analysis of the germination rates observed for both pollen samples at the lower concentration level and for the lower exposition time shows that a significative decrease is also

observed. This result shows that the pollen of both plants is affected by a concentration of air pollutant considered safe for human health.

Measurable effects of the *in vivo* and *in vitro* exposition of pollen to air pollutants are known (Wolters and Martens 1987). However, pollen expositions to low concentration of air pollutants at levels considered safe for humans are not common. Studies with pollen from *Crocus vernus* (Iridaceae) observed similar germination results to those observed in this work (Chichiricco and Picozzi 2007). Indeed, this work reported a 50% reduction on the germination rate when pollen was independently exposed to 0.5 ppm CO, 0.3 ppm O_3 and 0.2 ppm NO_2, and lower germination rates were calculated when the air pollutant levels were raised to 25 ppm CO, 0.5 ppm O_3 and 2 ppm NO_2. In another *in vitro* study a reduction on the germination rate of the tobacco pollen when exposed to 0.1 ppm O_3 for five hours and thirty minutes (Feder 1968). The same conclusion was obtained *in vitro* for *Lepidium virginicum* L. pollen when it was exposed to 0.6 ppm SO_2 for several hours (Du Bay and Murdy 1983).

The results now obtained for *Ostrya* and *Carpinus* pollen show that even at relatively low pollutants concentration, considered safe for human health, CO, O_3 and SO_2 provoke a significant reduction on the viability and germination. In the viability it was observed that O_3 and CO have a major negative effect on the *Ostrya* pollen and O_3 for *Carpinus* pollen. For the germination rate, SO_2 has a negative effect on the *Ostrya* pollen and O_3 and SO_2 have a negative effect on the *Carpinus* pollen. These different and specific effects of the air pollutants on the pollen of the two plants may be related with the different chemical reactivity of each gas and to the different characteristics of each plant (Omasa et al. 2002). A similar result was obtained for *Pinus nigra* pollen where the viability and germination was differently affected by O_3, NO_2 and CO (Gottardini et al. 2004).

Total soluble proteins and SDS-page

The TSP of the *Ostrya* and *Carpinus* pollen before and after exposure to the three air pollutants are shown in Table 2 and the ANOVA results of the air pollutant exposition effect are shown in Table 3. The analysis of these tables shows that the pollen exposition to the three air

pollutants affects the amount of TSP but only for *Ostrya* pollen this effect is statistically significant. A general decreasing trend of TSP with increasing pollen exposition is usually observed.

Previous studies showed that air pollutants are a stress factor for plant species affecting physiological processes in pollen (Helander and Savolainen 1997). Similar results were observed in *Acer negundo* pollen when exposed *in vitro* to SO_2 e NO_2 - a reduction trend was observed for exposed samples (Sousa et al. 2012). Also, the same effect was observed for *Cocos nucífera* and *Datura metel* pollen when exposed to SO_2 for 72 hours (Santra et al. 1991) – the reduction extent increased with the time and pollutant concentration exposition.

The analysis of the SDS-Page of both pollens (Fig. 2) show a polypeptide profile ranging from 15 to 130 kDa in the *Ostrya* samples and from 15 to 70 kDa in the *Carpinus* samples. Similarly to previously discussed about the TSP, the polypeptide profile of both pollen samples is not affected by the pollen exposition to the three air pollutants.

Previous research work with *Acer negundo* pollen (Sousa et al. 2012) showed a similar result, i.e. no clear differences were observed between the polypeptide profiles of exposed and non-exposed pollen samples. However, Majd et al. (2004) have detected the disappearance of selected polypeptides bands in the *Canna* pollen exposed to polluted atmosphere of Teheran. Also, Santra et al. (1991) have observed a significant decrease in the intensity of some electrophoretic bands of *Cocos nucífera* and *Datura metel* pollen exposed to SO_2. These results suggest that air pollution may affect protein expression and other biochemical processes in pollen (Cortegano et al. 2004).

The effect of air pollutants on the pollen polypeptide profile is a subject that deserves further research. In this work the results are not conclusive probably because to short exposition time were used to obtained reproducible measurable modifications. Future research should focus on the cellular and molecular mechanisms of action of air pollutants on pollen as well as on the modifications observed on the structure and protein content.

Conclusions

Ostrya and *Carpinus* pollen exposed to relatively low levels of atmospheric pollutants CO, O_3 and SO_2 observed a significative reduction of its viability and germination rates. Also, the total soluble proteins of both pollen samples were affected by the exposition to air pollutants but only *Ostrya* pollen show a statistically significant result.

Taking into consideration that this study was performed using relatively small concentrations of air pollutants, considered safe for human health, a discussion about these levels should be launch. Indeed, the modifications that pollen undergo upon air pollutants exposition can affect the human allergy potential of these biopollutants which, in urban centers where there exists high density of traffic and population, a correlation with the increase of asthma related diseases could be hypothesized.

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Table 1 – Average and standard deviation of the concentration of CO, O_3 and SO_2, temperature and relative humidity during the *Ostrya* spp and *Carpinus* spp pollen exposure experiments (+1d, 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).

Pollutant	Experiment	Concentration (ppm)		Temperature (^oC)		Relative humidity (%)	
		Ostrya	*Carpinus*	*Ostrya*	*Carpinus*	*Ostrya*	*Carpinus*
CO	+1d	9.73(0.75)	9.78(1.42)	24(2)	25(1)	48(1)	63(1)
CO	++1d	26.60(2.69)	27(2.54)	25(2)	25(1)	65(1)	69(2)
CO	++2d	26.85(1.84)	7.85(2.75)	25(1)	26(1)	54(2)	63(2)
O_3	+1d	0.061(0.014)	0.062 (0.021)	25(2)	24(1)	56(1)	48(1)
O_3	++1d	0.183(0.018)	0.183(0.023)	25(2)	25(2)	58(1)	62(1)
O_3	++2d	0.184(0.078)	0.183(0.026)	25(1)	24(1)	48(1)	48(1)
SO_2	+1d	0,13(0,02)	0.14(0.05)	24(1)	24(2)	59(1)	63(2)
SO_2	++1d	0,39(0,11)	0.39(0.12)	23(2)	24(1)	52(1)	55(1)
SO_2	++2d	0.40(0.15)	0.38(0.10)	25(2)	25(1)	55(1)	59(1)

* *Standard deviations in parenthesis*

Table 2 – Results of the viability, germination and total soluble protein (TSP) content of *Ostrya* and *Carpinus* pollen exposed to CO, O_3 and SO_2 (+1d, 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). *

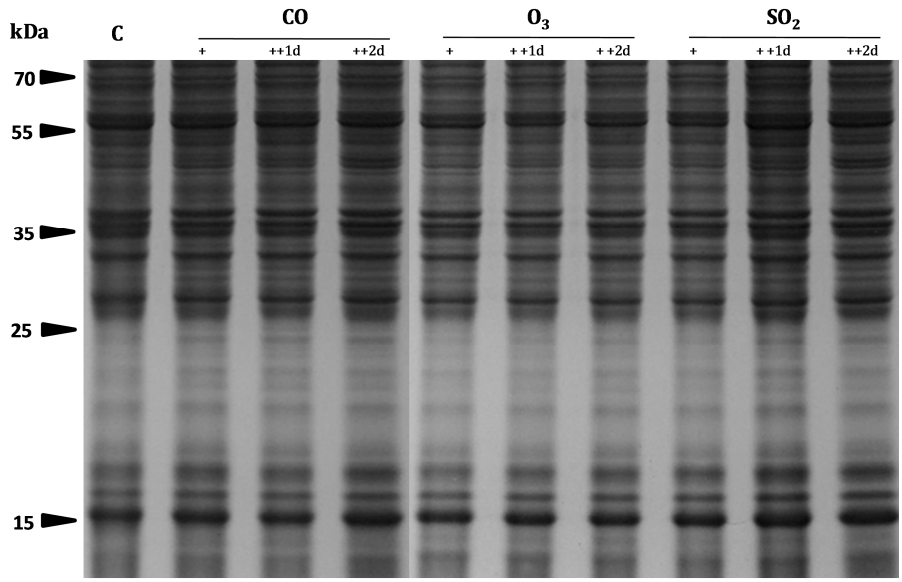
Pollutant	Experiment	Viability %		Germination %		TSP µg/Ml	
		Ostrya	*Carpinus*	*Ostrya*	*Carpinus*	*Ostrya*	*Carpinus*
Control	C	96(2)	97(2)	43(1)	36(1)	5256(65)	2891(45)
CO	+1d	91(1)	93(1)	39(1)	31(1)	4323(75)	2779(260)
CO	++1d	89(2)	90(1)	38(2)	29(2)	4149(19)	2831(74)
CO	++2d	86(1)	89(2)	36(1)	30(2)	3323(28)	2619(34)
O_3	+1d	90(2)	88(1)	36(1)	30(1)	3983(9)	2739(124)
O_3	++1d	86(1)	87(1)	34(1)	27(2)	3989(123)	2419(176)
O_3	++2d	84(2)	81(2)	30(4)	23(1)	4589(19)	2847(322)
SO_2	+1d	94(2)	91(1)	25(1)	25(1)	4546(14)	2287(198)
SO_2	++1d	93(1)	89(2)	24(1)	20(2)	4499(33)	2659(294)
SO_2	++2d	89(2)	86(1)	22(1)	20(1)	3953(24)	2371(23)

**Standard deviations in parenthesis*

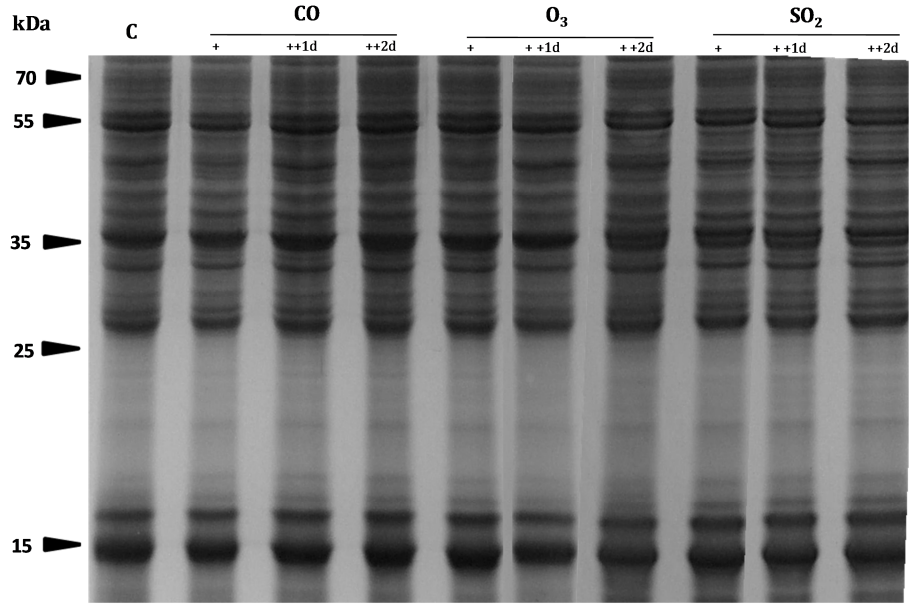
Table 3 - Effect of the *in vitro* exposure to pollutant gases (CO, O_3 and SO_2) of *Ostrya* and *Carpinus* given as *F* values of the analysis of variance (ANOVA).*

Property	Total *df*	F_{crit}	*F*		
			CO	O_3	SO_2
Ostrya spp					
Germination	19	3.2	29.3	36.6	393.9
Viability	23	3.1	84.8	107.9	27.1
Protein	7	6.6	449.2	147.9	368.6
Carpinus spp					
Germination	19	3.2	19.5	58.0	170.3
Viability	23	3.1	59.0	163.2	93.3
Protein	7	6.6	1.4	4.6	4.8

Fig. 1 – *Ostrya* (left) and *Carpinus* (right) plants.



a.



b.

Fig. 2 – SDS-PAGE of soluble protein of *Ostrya (a) and Carpinun (b)* pollen extracts exposed to CO, O_3 and SO_2. Signs above the lanes represent gas concentration level. 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.

Capitulo V

Efeitos de NO_2 na Viabilidade, Germinação, Conteúdo Proteico e Perfil de Polipeptídeos do Pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus*

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Effect of air pollutant NO_2 on *Betula pendula, Ostrya carpinifolia* and *Carpinus betulus* pollen

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Environmental Context. Asthma or allergic sensitization in humans is being associated with pollen and air pollution exposition. Moreover, when proteins like those existing on pollen, are exposed to polluted air containing nitrogen oxides, nitration processes occurs which increases the allergenic potential of pollen grains. In this context we have studied the effect low levels of nitrogen dioxide (NO_2), considered save for human life, on three birch trees pollen. As indicators of significant modifications on pollen induced by NO_2 we have measured the viability and germination rates and total soluble proteins. Results showed that pollen is significantly affected even when exposed at relatively low NO_2 air concentration.

Abstract. Pollen of *Betula pendula, Ostrya carpinifolia* and *Carpinus betulus* were exposed *in vitro* to two levels of NO_2 (about 0.034 and 0.067 ppm) - both below current atmospheric hour-limit value acceptable for human health protection in Europe (0.11 ppm for NO_2). Experiments were performed under controlled artificial solar light, temperature and relative humidity. The viability, germination and total soluble proteins of all the pollen samples exposed to NO_2 decreased significantly when compared with the non-exposed. The polypeptides profile of all the pollen samples show bands between 15 and 70 kDa and the exposition to NO_2 did not provoked any detectable changes in these profiles. The results obtained in this work showed that for the three plants under investigation the pollen exposition to NO_2 atmospheric levels considered safe for human health provoked significant modifications in the viability, germination and total soluble protein content.

Additional keywords: Air pollutants; viability; germination; soluble proteins.

Introduction

Pollen is a biological structure which role is the sexual reproduction of plants. Usually, urban tree planting is sought to promote air quality and respiratory health. However, pollen is being associated with asthma or allergic sensitization in humans and short term variation in pollen concentration has been correlated with allergy medication purchases and asthma symptoms [1-4]. Traffic-related air pollutants, like the nitrogen oxides, appear to enhance allergic diseases when compared with sulfur oxides air pollutants [5].

Urban air pollutants, like nitrogen dioxide (NO_2), can interact with pollen provoking fertility and protein content modifications affecting the reproductive cycle of spermatophytes [6-8]. At cellular level, air pollutants can provoke modifications on membrane structures, interfering with cellular mechanisms as well as on gene expression [9-11]. The wall of pollen has antioxidants, like the carotenoids, that react with reactive oxygen species with potential to reduce and repair the negative effect of air pollutants [12]. The study of the effect of atmospheric pollutants on pollen is crucial for the comprehension of the impact of atmospheric pollutants on pollen fertility and corresponding protein profiles, as well as on the reported increase of the prevalence allergies induced by pollen [3,13].

Pollen grains contain a relatively high amount of protein material which account for up to 5% of urban air particulate matter [14]. There is experimental evidence that during the inflammatory biological process nitrogen dioxide can promote the nitration of proteins that leads to the formation of 3-nitrotyrosine residues [5,14]. Although the purpose of this nitration process is not clear there are evidences that this will boost the immune response [5]. Also, it has been observed that proteins are nitrated when exposed to polluted air [15]. These evidences strongly suggest that nitrogen oxides present in polluted air may contribute to the increase of allergic diseases [5,15,14].

Nitrogen oxides are common pollutants to all high traffic urban centers and, depending on atmospheric conditions, more or less severe photochemical smog episodes can develop. The adverse effect of air pollutants on pollen occur during the germination and it was suggested that the oxidation of these gases provoke the inactivation of some critical factors involved on pollen [16].

From the metabolic point of view the critical factor cellular respiration [17,18]. Here, NO_2 and its nitrogenated derivatives have the affinity to bound with mitochondrial cytochromes and block the respiratory chain [19,7,17]. At molecular level, NO_2 and other air pollutants react with biomolecules, like proteins, lipids and nucleic acids, which are the main pollen reserve material, interfering with the pollinic tube development [20,21,7,22].

This paper shows the results of the assessment of the effect of relatively low *in vitro* NO_2 exposure of the pollen of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus*. The pollen properties that were evaluated were the viability, germination and soluble proteins. These three plants were chosen because its pollen is highly associated with respiratory human health problems in Europe [23-27]. Moreover, this research focuses on NO_2 levels that are considered safe for humans in order to assess potential synergistic effects between NO_2 pollution and pollen.

Experimental

Pollen samples

Betula pendula, *Ostrya carpinifolia* and *Carpinus betulus* are ornamental trees that produce staminate flowers [28]. Anthers were collected during the flowering season (first days of spring) from the Botanic Garden and City Park of Porto and were dried at 27°C. Pollen were released by gently crushed the anthers and passed through 63 µm sieves to isolate the pollen samples that were stored at –20 °C.

In vitro pollen exposition to NO_2

Pollen samples were *in vitro* exposed in a chamber made of acrylic and wood with dimensions about 50 x 70 x 50 cm and previously described [13]. In this chamber, sunlight was simulated by a Solar Simulator (Newport Oriel 96000 150 W) with Air Mass Filters 1.5 Global 81094 and a liquid light guide positioned 15 cm above the sample holder. A fan (SUNON SF23080AF) was used to homogenize the air inside the chamber. Air temperature and relative humidity were continually monitored using an EBRO EBI20 sensor. The gas concentrations were

monitored throughout each assay by AEROQUAL Series 500 sensors, registering data every minute. These sensors were connected to an external computer.

Each pollen sample [300 mg of dry weight (DW)] was exposed to NO_2 under two different concentrations - both below the current atmospheric hour-limit value acceptable for human health protection in Europe (0.11 ppm for NO_2) (European Union Directive 2008/50/EC of 21 May 2008 on ambient air quality and cleaner air for Europe). Two expositions times were used: six hours assays for both concentration levels (+ and ++1d); and, two days assays for the highest level (++2d). Pollen samples were placed into a Falcon tube with the bottom cut off and 23 µm grids (SEFAR PET 1000) were placed on both sides – this sampling system allows the circulation of the air through it and the re-suspension of the pollen.

NO_2 (≥ 99.5% v/v) concentrations were attained by injecting small volumes of gas directly obtained from bottles of compressed gas (Sigma-Aldrich). Three injections of gas per assay were performed using a micro syringe through septa placed at the front chamber wall within each 2h interval in order to prevent greater concentration fluctuations. Non-fumigated pollen samples were used as control. The average and standard deviations of the concentrations of NO_2, temperature and relative humidity observed in all experiments are shown in Table 1.

Pollen viability

Pollen viability was estimated using the trypan blue stain, which is a vital dye and its reactivity is based on the fact does not interact with the cell unless the membrane is damaged. So, all the non-colored cells are viable. Pollen grains (2 mg) are placed for 5 min in 0.5 ml of 60% trypan blue and centrifugation for 3 min at 13 200 rpm. Then 20 µl were placed on a microscope slide and observed at light microscope (DMLB: Leica). Pollen viability was determined as the percentage of non-colored 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.

Pollen germination

The germination of the exposed and non-exposed pollen samples occoured at 27 °C in the dark for 24 hours using the following optimized culture mediums: *Betula pendula*: 200 ppm H_3BO_3, 400 ppm L-proline, 15% sucrose and 0.5% agar; *Ostrya carpinifolia*: 200 ppm H_3BO_3, 200 ppm $CaCl_2$, 400 ppm L-proline, 25% sucrose and 0,5% agar; *Carpinus betulus*: 200 ppm H_3BO_3, 400 ppm $Ca(NO_3)_2 \cdot 4H_2O$, 400 ppm L-proline, 20% sucrose and 0,5% agar. Two replications were performed for each sample and five random fields were counted (100 pollen grains per field) using a light microscope (Leica DMLB). A pollen grain was classified as germinated when the pollinic tube was longer than the pollen size [29].

Extraction and quantification of soluble proteins

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 4 h. The suspension was centrifuged at 13200 rpm for 30 min at 4 °C. The supernatant was filtered through a 0.45 µm Millipore filter and centrifuged once again. The soluble protein content of all pollen extracts was quantified colorimetrically with the Coomassie Protein Assay Reagent (Pierce) by the Bradford Method [30].

SDS-PAGE

SDS-PAGE was performed as described by [31] in 12.5% (w/v) polyacrylamide gels under non-reducing conditions and the proteins were visualized by Coomassie Brillant Blue R-250 staining. The molecular mass of polypeptide bands was estimated by comparison with protein markers (PageRuler Plus Prestained Protein Ladder, Fermentas).

Results and discussion

Pollen viability

Table 2 shows the pollen viability before and after NO_2 exposition. A global analysis of this table shows that the viability of the pollen of the three plants decrease upon exposition to the

air pollutant. Indeed, while the viability percentage of the non-exposed *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* are respectively 93±1, 96±2% and 97±2%, the viability percentage of the exposed pollen (to the highest pollutant concentration – about 0.067 ppm NO_2) is about 80%. The analysis of variance (ANOVA) of the effect of the NO_2 exposition on the pollen viability (Table 2) shows that it is statistically significant for the three plants (the calculated *F* value is always higher than the F_{critic}).

The viability of the pollen of *Betula pendula* and *Carpinus betulus* are the most affected by the NO_2 exposure. Indeed, for these two plants the pollen shows a marked decrease on the viability even for the lowest pollutant exposition (non exposed *vs* exposed to about 0.034 ppm NO_2): *Betula pendula* - 93±1% *vs* 85±1%; *Carpinus betulus* - 97±2% *vs* 91±2%.

Pollen germination

The results of the *in vitro* germination of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus pollen before and after NO_2 exposition* are shown in table 2. The global analysis of the germination results show that a significative decrease of the germination of the exposed pollen of the three plants was observed. Indeed, while the germination percentage of the non-exposed *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* are respectively 39±1, 43±1% and 36±2%, the viability percentage of the exposed pollen (to the highest pollutant concentration) are, respectively, 21±1, 24±1% and 25±2%. The analysis of variance (ANOVA) of the effect of the NO_2 exposition on the pollen germination (Table 2) shows that it is statistically significant.

The exposition of the pollen of the three plants to the lower level of NO_2 has a similar effect to the germination as discussed to the viability, i.e. it has a marked reduction effect: *Betula pendula* - 39±1*vs* 26±2%; *Ostrya carpinifolia* - 43±1% *vs* 29±1%; and, *Carpinus betulus* - 36±2% *vs* 32±2%. These results support that even at relatively small NO_2 air pollutant concentration measurable effects are detected on the pollen of these plants.

The above results are similar to those observed for *Delonix regia* and *Peltophorum inermi* pollen where a marked reduction in its viability and germination was observed upon exposion to the polluted atmosphere, from industry and traffic, of Indore city [32]. In another *in vitro* study a

significant decrease in the *Populus deltoides*, *Pinus resinosa*, *Pinus nigra* and *Picea pungens* pollen was observed when exposed to 0.75 ppm of SO_2 [33]. The same conclusion was obtained in the in vitro study by Amjad and Shafighi [34] where they observed a significant reduction on the germination of *Chenopodium álbum* L. pollen exposed to polluted areas with SO_2, NO_2 and CO.

Total soluble proteins

The TSP of the *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen before and after exposure to NO_2 is shown in Table 2. The analysis of this table show that the soluble protein content of the exposed pollen significantly decreases when compared with the non-exposed samples ($F > F_{crit}$). Also, this decreasing trend is most significant for *Ostrya carpinifolia* pollen ($F = 280.6 > F_{crit} = 6.6$) - *Betula pendula* - $F = 7.8 > F_{crit} = 4.1$; and, *Carpinus betulus* - $F = 19.4 > F_{crit} = 6.6$.

A significant decrease on the TSP has been observed for *Ricinus communis* pollen when exposed to SO_2 and NO_2 and the variation was enhanced when the concentration and exposition time increased [35]. These results show that air pollutants are stress factors that can affect the expression of protein which constitute the plant defenses provoking several structural and physiological modifications at cellular and molecular level as reported for the *Cassia glauca* Lamk pollen when exposed to urban air pollution in India [36].

As previously discussed, the amount of soluble protein content of the pollen exposed to NO_2 showed significative variation relatively to the non exposed pollen and SDS-page was used to observe if changes in the polypeptide profile between exposed and non-exposed pollen samples also exist (Fig. 1). Indeed, no clear changes among all the electrophoretic profiles were detected but that they revealed several polypeptide bands ranging from 70 to 15 kDa after Coomassie staining of the gel. The polypeptide profiles of all the pollen samples showed five clusters of bands about of 70, 55, 35, 25 and 15 kDa. This result is similar to other studies that did not reported measurable differences in the polypeptide profiles between exposed and non-exposed air pollutants [37]. However, other studies observed the disappearance of some bands corresponding to 25 and 45 kDa in *Canna* pollen exposed to air pollution in Teheran [38] and to a significative decrease of some

polypeptides bands in *Lagerstroemia* pollen exposed to SO_2, NO_2 and CO for ten and twenty days in air polluted areas of Teheran [39]. These results suggest that the exposition of pollen to air pollutants may provoke measurable SDS-page differences between the exposed and non-exposed samples but relatively high exposition and/or high concentration of pollutants must exist. In this work the time of exposure to NO_2 and the relatively small gas concentration were not sufficient to provoke marked modifications on the polypeptide profiles.

Conclusions

The short time exposition of the pollen of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* to relatively small concentrations of NO_2 (below current atmospheric hour-limit value acceptable for human health protection in Europe) provoked significant decrease on the viability percentages, germination rates and soluble proteins. Taking into consideration that the results were obtained under air NO_2 concentrations considered safe for human health, a discussion about these levels should occur and further research about the cellular and molecular mechanisms of the NO_2 effect is necessary. Also, the synergistic effect between pollen (included in the bioaerosol class) and atmospheric air pollutants should be further analyzed and the correlation with the increase of allergic sensitization discussed.

The significant decrease of TSP observed in this work show that NO_2 affected the pollen proteins. This result supports the above discussion about the nitration of proteins that may occur in the presence of nitrogen oxides and in the increased allergenicity of the pollen exposed to this type of air pollutants.

Acknowledgments

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Table 1 – Average and standard deviation of the concentration of NO_2, temperature and relative humidity during the *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus* pollen exposure experiments (+ - low level; ++ - high level during one day; 2d++ – high level during two days).

Level		+	++	2d++
Concentration (ppm)	*Betula*	0.035(0.007)	0.066(0.009)	0.060(0.008)
	Ostrya	0,034(0,007)	0,067(0,009)	0,068(0,014)
	Carpinus	0,033(0,004)	0,068(0,011)	0,068(0,009)
Temperature (^{o}C)	*Betula*	29(1)	30(1)	30(1)
	Ostrya	24(2)	24(2)	25(1)
	Carpinus	23 (2)	24(2)	25(1)
Relative humidity (%)	*Betula*	59(1)	63(1)	61(1)
	Ostrya	50(1)	56(1)	54(1)
	Carpinus	48(1)	54(1)	58(1)

**Standard deviations in parenthesis*

Table 2 – Results of the viability (V), germination (G) and total soluble protein (TSP) content of *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus* pollen exposed to NO_2. Signs represent gas concentration level, namely: + - low level; ++ - high level during one day; 2d++ – high level during two days. *

Level	*Betula pendula*			*Ostrya carpinifolia*			*Carpinus betulus*		
	V %	G %	TSP µg/mL	V %	G %	TSP µg/mL	V %	G %	TSP µg/mL
C	93(1)	39(1)	1238(355)	96(2)	43(1)	5256(65)	97(1)	36(1)	2891(45)
+	85(1)	26(2)	572(18)	93(1)	29(1)	3923(19)	88(1)	31(1)	2727(209)
++	82(1)	23(1)	788(14)	92(2)	26(1)	2966(146)	89(1)	29(2)	2079(107)
2d++	80(1)	21(1)	1017(14)	88(2)	24(1)	4416(5)	84(1)	30(2)	2247(62)
F – value (F_{crit})	177.8 (3.1)	144.9 (3.2)	7.8 (4.1)	59.8 (3.1)	190.9 (3.2)	280.6 (6.6)	163.2 (3.1)	19.5 (3.2)	19.4 (6.6)

**Standard deviations in parenthesis*

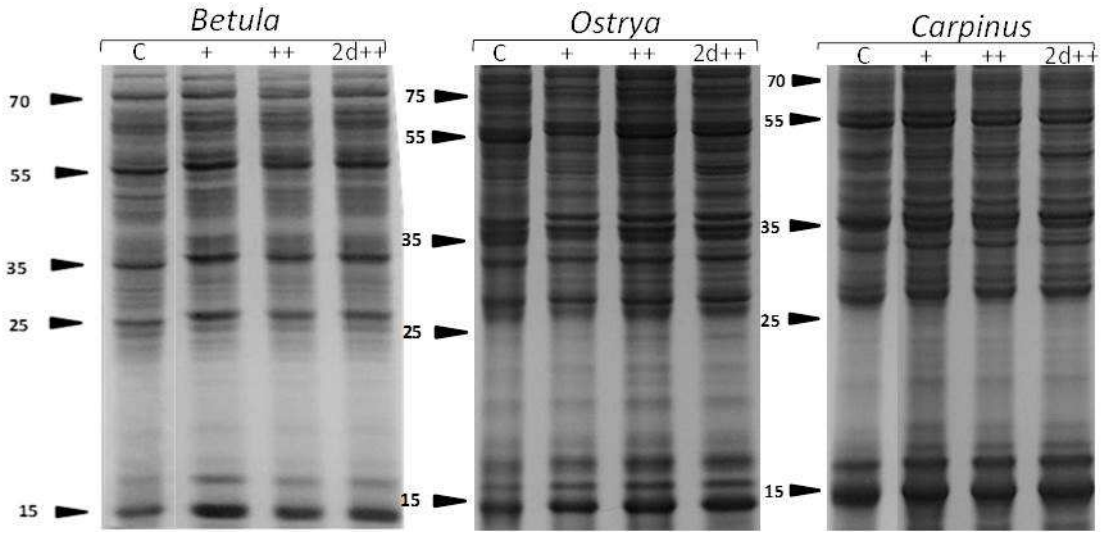

Fig. 1 – SDS-PAGE of total soluble proteins of *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus* pollen extracts exposed to NO_2. Signals above the lanes represente gas concentration level: C – control, + - low level, ++ - high level during one day and 2d++ - high level during two days.

Capitulo VI

Efeitos de CO, O_3, SO_2, e NO_2 na Alergenicidade do Pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus*

6. Effects of atmospheric pollutants (CO, O_3, SO_2 and NO_2) on the allergenicity of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen

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Aerobiologia

Effects of atmospheric pollutants (CO, O3, SO2 and NO2) on the allergenicity of Betula pendula, Ostrya carpinifolia and Carpinus betulus pollen

--Manuscript Draft--

Manuscript Number:	
Full Title:	Effects of atmospheric pollutants (CO, O3, SO2 and NO2) on the allergenicity of Betula pendula, Ostrya carpinifolia and Carpinus betulus pollen
Article Type:	Original paper
Keywords:	Allergic sensitization; Immunoblotting; Betula pendula pollen; Ostrya carpinifolia pollen; Carpinus betulus pollen; Air pollutants.
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Abstract:	Pollen of Betula pendula, Ostrya carpinifolia and Carpinus betulus was exposed in vitro to relatively low levels of the air pollutants namely carbon monoxide (CO), ozone (O3), sulfur dioxide (SO2) and nitrogen dioxide (NO2). The allergenicity of the exposed pollen was compared with that of non exposed pollen samples to assess if air pollution exposition affects the allergenicity potential of pollen. Pollen was exposed to two levels of the air pollutants about the current atmospheric hour-limit value acceptable for human health protection in Europe. Experiments were performed under controlled artificial solar light, temperature and relative humidity. The profile of polypeptides of all the pollen did not showed observable detectable changes in these profiles between the exposed and non-exposed samples. However, the immunodetection assays indicated higher IgE recognition by all sera of sensitized patients to the pollen protein extracts in all exposed samples in comparison to the nonexposed samples. The common reactive bands to the three pollen samples correspond to 58 and 17 kDa proteins. These results show that the Betula pendula, Ostrya carpinifolia and Carpinus betulus pollen exposition to low levels of CO, O3, SO2 and NO2 provoke increased allergic reaction to sensitized individuals.

Effects of atmospheric pollutants (CO, O_3, SO_2 and NO_2) on the allergenicity of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen

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Abstract

Pollen of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* were exposed *in vitro* to relatively low levels of the air pollutants carbon monoxide (CO), ozone (O_3), sulfur dioxide (SO_2) and nitrogen dioxide (NO_2). The allergenicity of the exposed pollen was compared with that of non exposed pollen samples to assess if air pollution exposition affects the allergenicity potential of pollen. Pollen was exposed to two levels of the air pollutants about the current atmospheric hour-limit value acceptable for human health protection in Europe. Experiments were performed under controlled artificial solar light, temperature and relative humidity. The polypeptides profile of all the pollen did not showed observable detectable changes in these profiles between the exposed and non-exposed samples. However, the immunodetection assays indicated higher IgE recognition by all sera of sensitized patients to the pollen extracts in all exposed samples in comparison to the nonexposed samples. The common reactive bands to the three pollen samples correspond to 58 and 17 kDa proteins. These results show that the *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen exposition to low levels of CO, O_3, SO_2 and NO_2 provokes increased allergic reaction to sensitized individuals.

Keywords: Allergic sensitization; Immunoblotting*; Betula pendula* pollen; *Ostrya carpinifolia* pollen; *Carpinus betulus* pollen; Air pollutants.

1 Introduction

Under contaminated environment pollen is exposed to levels of chemical substances that can induce modifications in the external wall as well as in the proteins associated with allergenicity. This chemical modified pollen may be responsible for the increase asthma and/or allergic sensitization in humans living in urban and industrial areas (Wyler et al. 2000; Gilles et al. 2009; Traidl-Hoffmann et al. 2009; Bosch-Cano et al. 2011).

The prevalence of pollinosis is increasing (about 40%) during the last two decades, particularly in industrialized countries, but the reasons for this trend are not straightforward (Traidl-Hoffmann et al. 2009; D'Amato et al. 2010). Several environmental and epidemiological studies showed that in the European Community countries, between 8 to 35% of the adults and youngsters have increased IgE sensitization to pollen allergens and this problem is more frequent in individuals leaving in urban and industrialized centers when compared with those living in rural areas (Majd, 2004; D'Amato et al. 2000). These observations suggest that air pollutants present at higher levels in urban centers can modify the chemical structure of proteins present in pollen provoking significant increases in the IgE response of exposed individuals (Emberlin, 1995; Devalia et al. 1998; D'Amato et al., 2007; Kim et al. 2013). In Europe pollinosis is usually caused by pollen from Grasses, Urticaceae, Betulaceae, Alder, Olive and Cypress (D'Amato and Spieksma 1991; Filon et al. 2000; D'Amato et al., 2007; Peternel et al. 2007).

The air contaminants carbon monoxide (CO), sulfur dioxide (SO_2) and nitrogen dioxide (NO_2) are mainly emitted to the atmosphere by the burning of fossil fuels, industrial activities and motorized traffic (Kammerbauer et al. 1987). Ozone (O_3) is a secondary air pollutant resulting for photochemical smog reactions that begin with nitrogen oxides and volatile hydrocarbons (Karlsson et al. 2007). Taking into consideration their origin these four air contaminants are always present in urban centers are their air concentration is usually monitored in populated areas to check if the corresponding levels follow national and/or international regulations proposed for human health protection.

In Porto (Portugal) the plantation of Betulaceae family trees for ornamental purposes is increasing in city avenues, street-walks and in public parks. Although this urban tree planting is sought to promote air quality and respiratory health, besides aesthetic purposes, currently there are increasing concerns about the increase allergenic impact on residents. Indeed, besides pollen, in city centers the increased concentration of air pollutants is usually directly responsible for the enhancement of allergic diseases and, the synergistic interaction of air pollutants and pollen may result in further human allergenic sensitization. The objective of this work was to study the effects of CO, O_3, SO_2 and NO_2 in the allergenicity of *in vitro* exposed *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* pollen. In order to assess the effect of air contaminants CO, O_3, SO_2 and NO_2 in the pollen the electrophoretic profiles of the protein extracts were acquired and the pollen allergenic changes were evaluated according to the binding affinity to specific IgE of atopic patient sera to pollen extracts (immunoblots).

2 Material and Methods

2.1 Pollen samples

Betula pendula, *Ostrya carpinifolia* and *Carpinus betulus* are ornamental trees that produce staminate flowers (Chen et al. 1999). Anthers were collected during the flowering season (first days of spring) from the Botanic Garden and City Park of Porto, were dried at 27 ºC, gently crushed and the pollen thus released was passed through different grades of sieves to obtain pure pollen. After, the isolated pollen was stored at –20 °C.

2.2 In vitro pollen exposition to CO, O_3, SO_2 and NO_2

Pollen samples were artificially exposed in a fumigation chamber made of acrylic and wood mix with dimensions about 50 x 70 x 50 cm and described in the literature (Sousa et al.

2012). Each pollen sample [300 mg of dry weight (DW)] was exposed individually with CO, O_3, SO_2 and NO_2, under two different concentrations levels – the low level was used for one day (+) and the high level for one (+) and two days (2d ++) (Table 1). Pollen samples were placed into a Falcon tube with the bottom cut off and 23 µm grids (SEFAR PET 1000) were placed on both sides – this sampling system allows the circulation of the air through it and the re-suspension of the pollen.

SO_2 (99.9% v/v) and NO_2 (≥ 99.5% v/v) concentrations were attained by injecting small volumes of gas directly obtained from bottles of compressed gas (Sigma-Aldrich). CO was generated by chemical reaction of sulfuric acid with formic acid– it was generated by mixing, in a test tube, stoichiometric amounts of the two concentrated acids and subsequently injected into the chamber. Three injections of gas per assay were performed using a micro syringe through septa placed at the front chamber wall within each 2h interval in order to prevent greater concentration fluctuations. Ozone was generated using an A2Z OZONE system coupled to an OMRON H3DK-S1 timer, controlling the injection of ozone for brief periods of time in order to offset the high ozone reactivity. Non-fumigated pollen samples were used as control. Table 1 shows the exposure experiments that were performed and the corresponding pollutants levels and environmental conditions.

2.3 Extraction and quantification of soluble proteins

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 4 h. The suspension was centrifuged at 13200 rpm for 30 min at 4 °C. The supernatant was filtered through a 0.45 µm Millipore filter and centrifuged once again. The soluble protein content of all pollen extracts was quantified colorimetrically with the Coomassie Protein Assay Reagent (Pierce) by the Bradford Method (Bradford, 1976).

2.4 SDS-PAGE and Immunoblots

Proteins from pollen extracts were separated in 12.5% polyacrylamide gels under reducing conditions (Laemmli, 1970), and the proteins were visualized by Coomassie Brilliant Blue R-250 staining. The molecular weight of protein bands was estimated by comparison with an established protein marker (PageRuler Plus Prestained Protein Ladder, Fermentas). For immunoblotting analysis, the protein was electroblotted onto nitrocellulose membranes (Protran, Whatman Schleicher and Schuell, Germany). The membranes were saturated during 1 h in a blocking solution (5% nonfat dry milk (w/v), 0.1% goat serum (v/v), in 20 mM Tris, 150 mM NaCl (TBS) and 0.1% Tween) and then incubated overnight at 4 °C with sensitized and nonsensitized patient sera to Betulaceae pollen diluted 1:10 in blocking solution for the identification of allergens. After washing, bound specific IgE were detected by horseradish peroxidase-conjugated antihuman IgE serum (Southern Biotechnology Associates). An ECL solution (Luminata Crescendo, Interface, Lda.) was used as a detection system. The chemiluminescent signal was exposed to AGFA medical X-ray film and developed by Fuji medical film processor model FPM 100A. The antigenic profile bands of the SDS-PAGE and immunoblots were quantified by the software MyImageAnalysis v1.1 (Thermo Fisher Scientific Inc.).

3 Results and discussion

3.1 Total soluble proteins

In order to check if the pollen exposition to the four air contaminants would affect the polypeptide profiles of the pollen extracts the corresponding electrophoretic profiles were obtained for the three pollen samples and compared with the non-exposed pollen (Fig.1). The analysis of this figure reveals no detectable difference among the exposed and non-exposed pollen samples. All the electrophoretic profiles have in common several bands ranging from 70 to 15 kDa and the most

clearly defined are: 70, 55, 35, 25, 15 kDa. This result shows that the exposition of pollen to the four air contaminants did not modified significantly (within the selectivity and sensitivity of the SDS-PAGE technique) the chemical properties of the aqueous pollen extracts, i.e. the water solubility and molecular weight of the soluble proteins. Nevertheless, other authors also reported no measurable differences in the polypeptide profiles of protein extracts of exposed and no exposed to air pollutants pollen (Rezanejad, 2007). However, Rezanejad et al. (2003) described a significant decrease in the intensity of several bands corresponding to soluble proteins from *Lagerstroemia indica* L. pollen exposed to SO_2, NO_2 and CO for ten and twenty days in the polluted areas of Tehran city. Similar results were described by Cortegano et al. (2004) which suggested that the exposition of pollen to air pollutants would induce structural chemical modifications in the pollen proteins. These different observations may result from different plant/pollen sensitivity to air pollutants and different concentrations of the chemical substances at which the plant/pollen were exposed.

3.2 Immunoblots

To assess the effects of the four air pollutant on the allergenicity of the three pollen samples under investigation, 24 immunoblots were assayed using different patient sera allergic to Betulaceae pollen (8 for each plant) – only 12 plots are shown in Fig. 2 (4 for each plant). Pollen allergenic changes were evaluated according the binding affinity to specific IgE which results in higher optical densities (Fig. 2).

Increased optical densities were observed in protein bands of the pollen exposed to the four air pollutants when compared with the non-exposed pollen (Fig. 2). These results demonstrate that the three Betula pollen samples contain multiple potential allergen proteins that can be modified upon exposition to low levels of CO, O_3, SO_2 and NO_2. The results obtained for the three pollen samples are:

- *Betula pendula* pollen show that among the 8 patient sera used, 100% reacted to protein bands around 58 and 17 kDa, 62.5% reacted to protein bands around 45, 35 and 15 kDa, 37.5%

reacted to protein bands around 27 and 24 kDa, 25% reacted to a protein band around 18 kDa and 12.5% reacted to a protein band around 20 kDa.

- *Ostrya carpinifolia* pollen show that among the 8 patient sera used, 100% reacted to protein bands around 60, 58, 35, 22 and 17 kDa, 87.5% reacted to protein bands around 29, 27, 24 and 15 kDa and 37.5% reacted to protein bands around 42 and 18 kDa.

- *Carpinus betulus* pollen show that among the 8 patient sera used, 100% reacted to protein bands around 58, 45, 35 and 31 kDa, 87.5% reacted to a protein band around 60 kDa, 62.5% reacted to protein bands around 29, 24 and 17 kDa, 37.5% reacted to protein bands around 42, 25, 22 and 20 kDa and 12.5% reacted to a protein band around 53 kDa.

The analysis of these results allow the detection of two reactive bands common to the three plants, namely at 58 and 17 kDa. Accordingly to Patriarca, et al. (2000) a 17 kDa protein is the main allergen present in the pollen of Betulaceae family and its reactivity towards IgE is due to the presence of Bet v1. The allergen with a molecular mass of 58 kDa, which is present in all the assayed sera was not yet characterized and identified as an allergen protein in the Betulaceae family pollen. Following EUROIMMUN data base (2008) allergens corresponding to 35, 24, 18, 17 e 15 kDa may correspond to antibodies already characterized and designed by Bet v6, Bet v3, Bet v7, Bet v1 and Bet v2, respectively.

3.3 The specific effect of the air pollutant

The analysis of the set of immunoblots obtained for each plant, taking into consideration the pollutant level and time of exposure shows some particularities:

- In the *Betula pendula* pollen the IgE reactivity was higher in allergens present in the pollen exposed to the higher pollutant level for two days for all the four pollutants.
- In the *Ostrya carpinifolia* pollen the IgE reactivity was higher in allergens present in the pollen exposed to the higher pollutant level for two days for O_3, SO_2 and NO_2 and to the higher pollutant level for both exposition times for CO.

- In the *Carpinus betulus* pollen the IgE reactivity was higher in allergens present in the pollen exposed to the higher pollutant level for two days for CO, SO_2 and NO_2 and to the higher pollutant level for both exposition times for O_3.

These results demonstrate that the pollen exposition to the four air pollutants under investigation increase the allergenicity and may induce higher IgE reactivity in patients allergic to the pollen of these plant species. Similar results were observed by Sousa et al. (2012) that measure high IgE reactivity to allergens in *Acer negundo* pollen after *in vitro* exposition to SO_2 and NO_2. Other authors (Behrendt et al. 1997) show that SO_2 and NO_2 increase the allergenic potential of pollen. Air pollutants provoke stress in plants which may induce the regulation of several protein correlated with pathogeneses and defense system as reported for *Cupressus arizonica* pollen exposed to the polluted atmosphere at Toledo (Spain) (Cortegano et al., 2004).

This study show that the pollen of *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* contains several protein potentially allergenic that can be modified by chemical substances present in the atmosphere. Bowler and Crapo (2002) suggested that O_3 can modified the chemical structure of the proteins present in pollen through the generation of reactive oxygen species, which can increase the allergenicity. NO_2 can increase the allergenic potential by the direct nitration of allergens (Franze et al. 2005; Gruijthuijsen et al. 2006). SO_2 and CO can affect the morphology of pollen and induce an increase of free aminoacids which may be responsible by an increase of the allergic symptoms in exposed individuals (Ruffin et al. 1986; Bist et al. 2004).

4 Conclusions

The exposition of pollen from *Betula pendula*, *Ostrya carpinifolia* and *Carpinus betulus* to air concentrations of CO, O_3, SO_2 and NO_2 close to the current atmospheric hour-limit value acceptable for human health protection in Europe provokes an increase of the pollen allergenicity, at least to atopic individuals. The allergens common to the three plants correspond to proteins with 58 and 17 kDa. Further research is need for the characterization of the here detected 58 kDa

allergen protein to assess the allergenic potential and to develop new immunoassays about Betulaceae pollen allergenicity.

The concentration levels of the air pollutants used in this work were around those considered safe for human health. However, as demonstrated, those levels provoked measurable immunological responses which would affect human health, at least of those with allergic diseases. Consequently, further research and discussion is necessary about the currently atmospheric limits considered safe for humans. This discussion should include the synergistic effects that results from the interaction of air pollutants and bioaerosols and the increase of allergic diseases prevalence.

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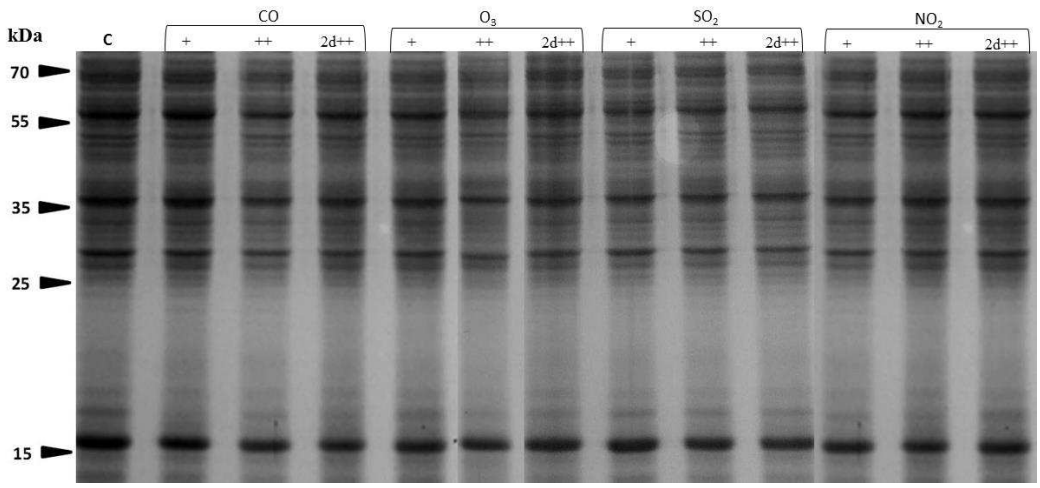
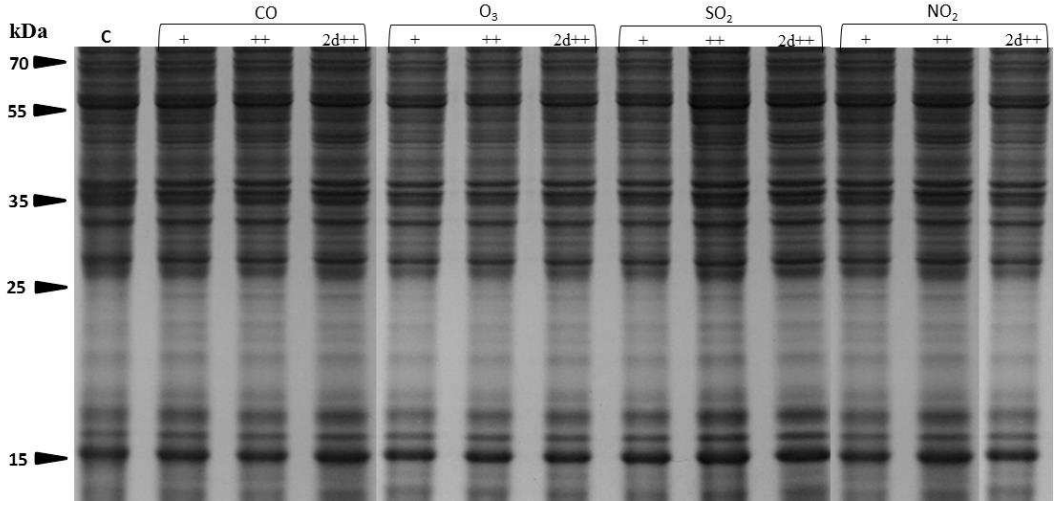
Table 1 – Average and standard deviation of the concentration of CO, O_3, SO_2 and NO_2, temperature and relative humidity during the *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus* pollen exposure experiments (+ - low level; ++ - high level during one day; 2d++ – high level during two days).

Polutant	Level	Concentration (ppm)			Temperature (^oC)			Relative humidity (%)		
		Betula	*Ostrya*	*Carpinus*	*Betula*	*Ostrya*	*Carpinus*	*Betula*	*Ostrya*	*Carpinus*
	+	9.77(1.28)	9.73(0.75)	9.78(1.42)	27(1)	24(2)	25(1)	71(1)	48(1)	63(1)
CO	++	30.98(3.97)	26.60(2.69)	27.00(2.54)	27(1)	25(2)	25(1)	68(1)	65(1)	69(2)
	++2d	23.05(3.38)	26.85(1.84)	27.85(2.75)	27(1)	25(1)	26(1)	65(1)	54(2)	63(2)
	+	0.061(0.017)	0.061(0.014)	0.062(0.021)	27(1)	25(2)	24(1)	70(1)	56(1)	48(1)
O_3	++	0.192(0.026)	0.183(0.018)	0.183(0.023)	27(1)	25(2)	25(2)	66(1)	58(1)	62(1)
	++2d	0.189(0.036)	0.184(0.078)	0.183(0.026)	27(1)	25(1)	24(1)	72(2)	48(1)	48(1)
	+	0.19(0.05)	0.13(0.02)	0.14(0.05)	27(1)	24(1)	24(2)	57(1)	59(1)	63(2)
SO_2	++	0.35(0.20)	0.39(0.11)	0.39(0.12)	29(1)	23(2)	24(1)	56(1)	52(1)	55(1)
	++2d	0.54(0.24)	0.40(0.15)	0.38(0.10)	29(1)	25(2)	25(1)	57(1)	55(1)	59(1)
	+	0.035(0.007)	0.034(0.007)	0.033(0.004)	29(1)	24(2)	23 (2)	59(1)	50(1)	48(1)
NO_2	++	0.066(0.009)	0.067(0.009)	0.068(0.011)	30(1)	24(2)	24(2)	63(1)	56(1)	54(1)
	++2d	0.060(0.008)	0.068(0.014)	0.068(0.009)	30(1)	25(1)	25(1)	61(1)	56(1)	58(1)

**Standard deviations in parenthesis*


kDa
C
CO
O3
SO2
NO2
+
++
2d++
70
55
35
25
15

a.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SO2
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+
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2d++
70
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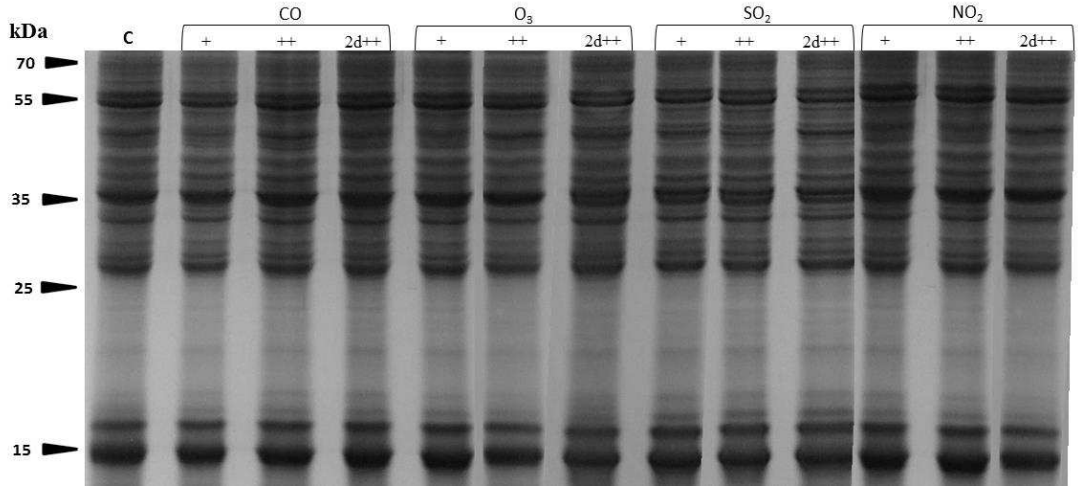
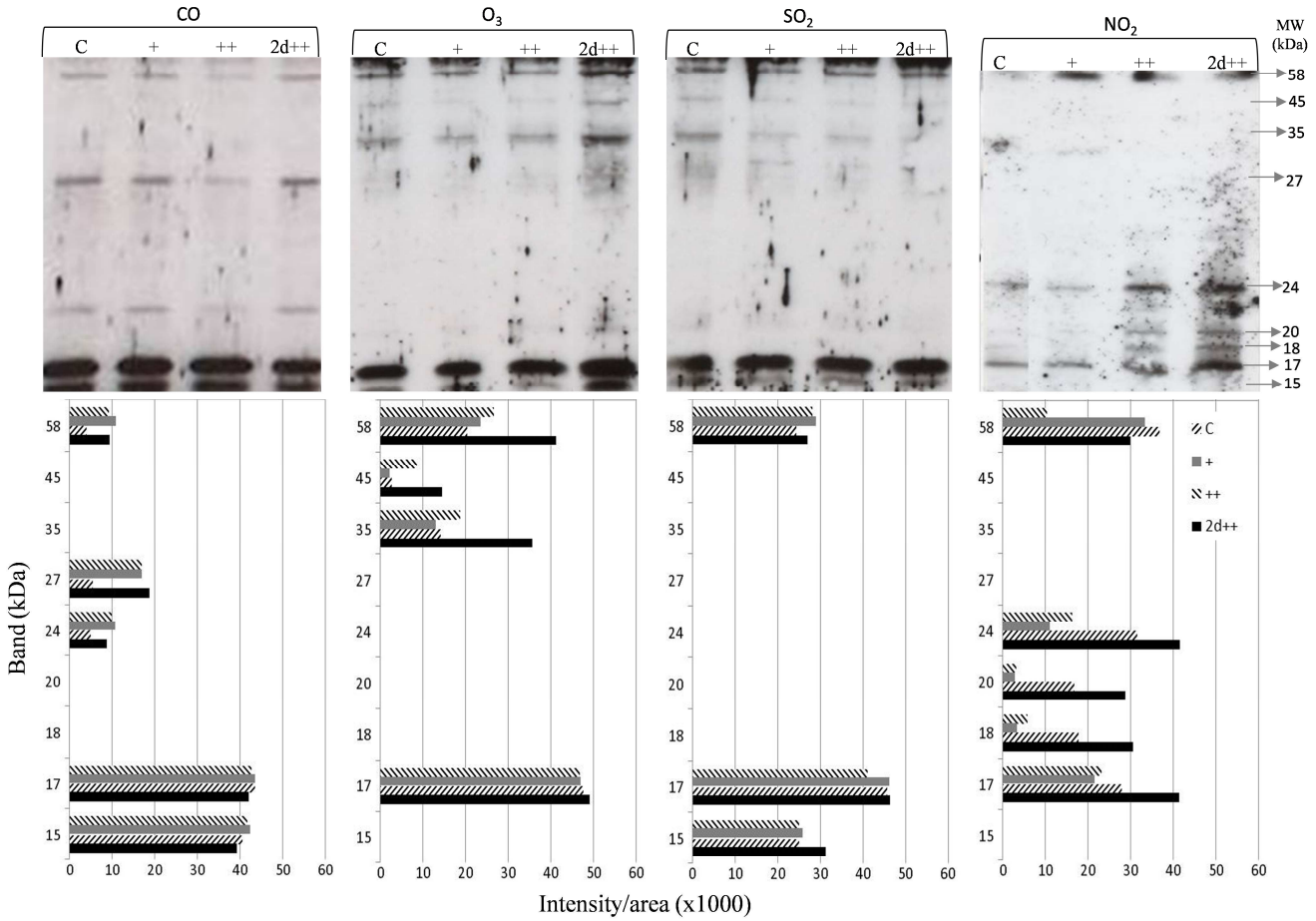
35
25
15

b.

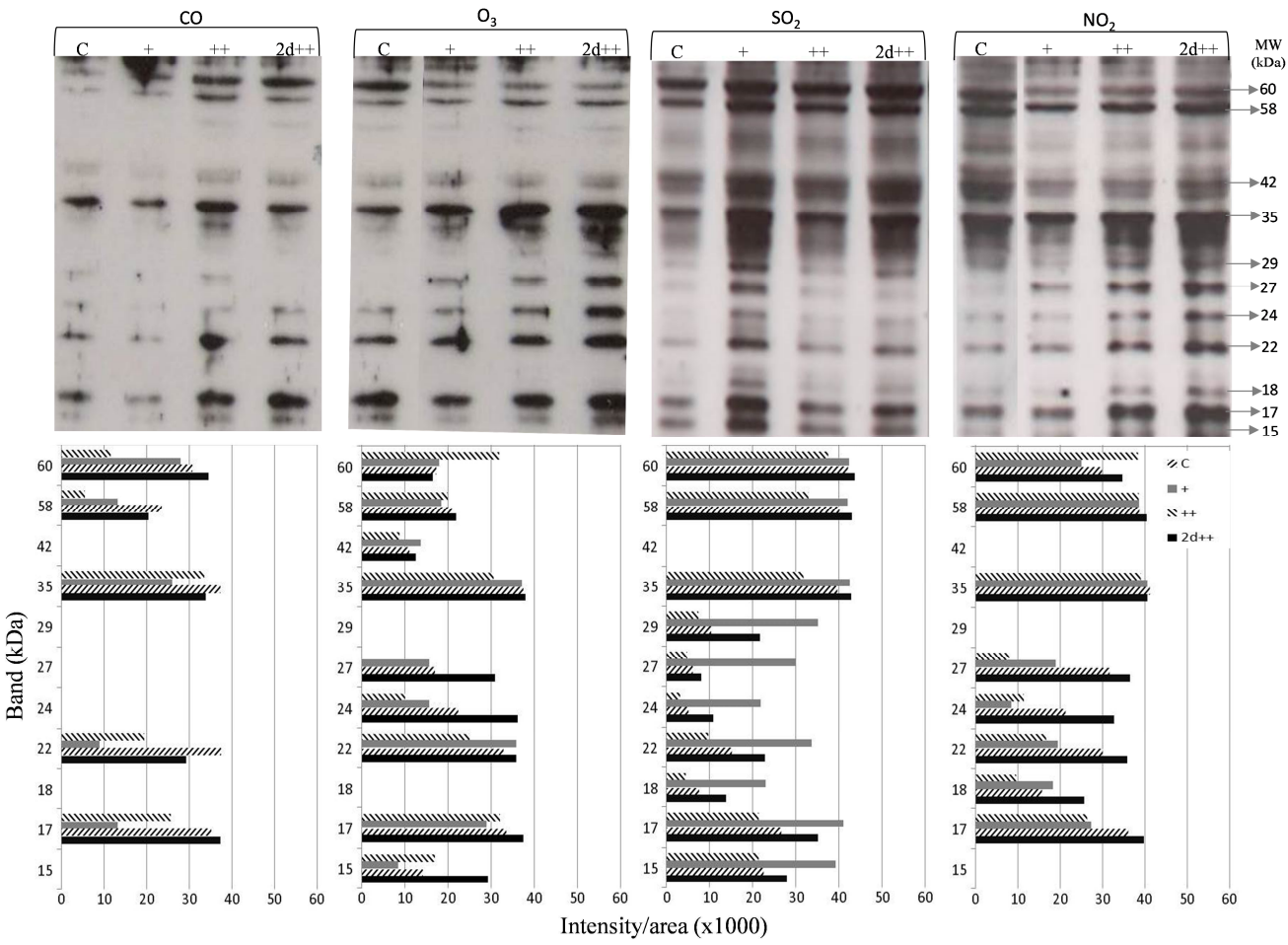

kDa
C
CO
O3
SO2
NO2
+
++
2d++
70
55
35
25
15

c.

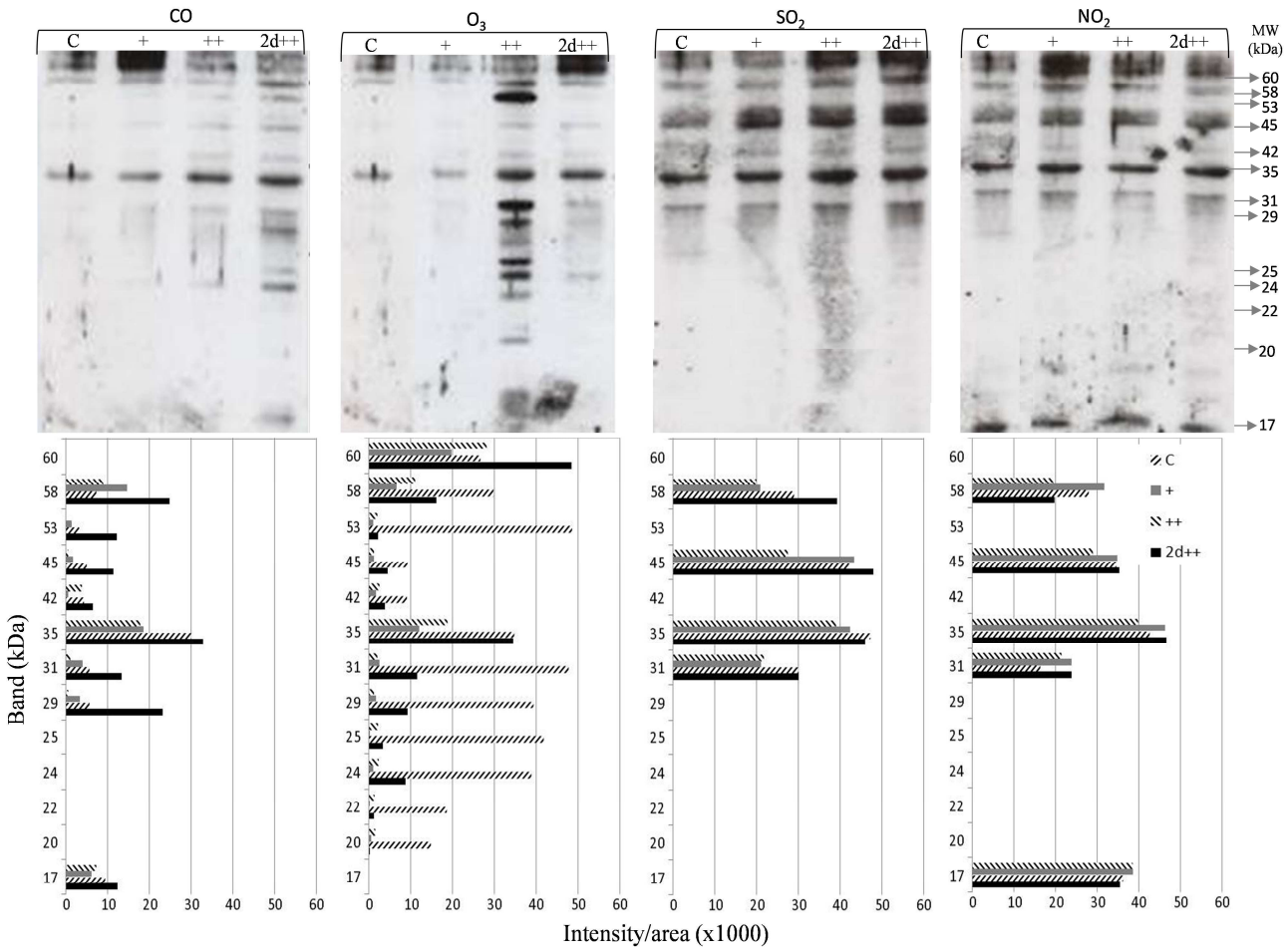
Fig. 1 SDS-PAGE of soluble protein of *Betula pendula* (a), *Ostrya carpinifolia* (b) *and Carpinus betulus (c)* pollen extracts exposed to CO, O_3, SO_2 and NO_2. Signs above the lanes represent gas concentration level. C – control; **+** - Low level; **++** - High level and **2d++** - High level two days of exposition.


CO
O3
SO2
NO2
C
+
++
2d++
MW (kDa)
58
45
35
27
24
20
18
17
15
Band (kDa)
Intensity/area (x1000)
0
10
20
30
40
50
60

a.


CO
O3
SO2
NO2
C
+
++
2d++
MW (kDa)
60
58
42
35
29
27
24
22
18
17
15
Band (kDa)
Intensity/area (x1000)
0
10
20
30
40
50
60

b.



c.

Fig. 2 Immunoblots and optic densities (O.D.) of IgE-reactive bands of *Betula pendula* (a), *Ostrya carpinifolia* (b) and *Carpinus betulus* (c) pollen extracts exposed to CO, O_3, SO_2 and NO_2. Signs above the lanes represent gas concentration level: **C** - control; **+** - Low level; **++** - High level and **2d++** - High level two days of exposition.

Capitulo VII

Conclusões e Perspetivas Futuras

7. Conclusões e Perspetivas Futuras

O pólen de plantas de família Betulaceae germinaram melhor em meios de cultura contendo H_3BO_3, $CaCl_2$ ou $Ca(NO_3)_2$, L-prolina e sacarose, embora as quantidades de alguns componentes sejam diferentes para a germinação do pólen das diversas plantas desta família. Assim, são ainda necessários mais estudos para se encontrar uma única proporção dos componentes estudados nesta pesquisa, capaz de proporcionar melhor germinação do pólen de Betulaceae.

O pólen de *Betula pendula*, *Ostrya carpinifolia* e *Carpinus betulus* exposto *in vitro* a CO, O_3, SO_2 e NO_2 em concentrações considerados seguros para a proteção da saúde humana na Europa, mostraram uma redução significativa nas taxas de viabilidade e germinação, bem como no conteúdo das proteínas solúveis das amostras do pólen, quando comparado com as amostras do pólen não exposto. Contudo, estes poluentes não revelaram diferenças mensuráveis entre os perfis polipeptídicos de extratos proteicos do pólen exposto e não exposto.

Concentrações de CO, O_3, SO_2 e NO_2 nos valores-limite para proteção de saúde humana na Europa, podem aumentar a alergenicidade do pólen destas espécies arbóreas, pondo em risco a saúde da população, especialmente dos indivíduos atópicos. As bandas reativas comuns correspondem a proteínas de 58 e 17 kDa. Assim, são necessários mais estudos de biologia molecular, baseados na espectrometria de massa e RT-PCR, para identificar a proteína alergénica de 58 kDa verificada nesta pesquisa, de modo a estimar-se o seu potencial alérgico e facilitar a obtenção de proteínas recombinantes para futuros imunoensaios sobre alergenicidade do pólen de Betulaceae, pois, a proteína de 17 kDa já é conhecida como principal alergénio do pólen das plantas desta família.

Levando em consideração que estes resultados foram obtidos em concentrações próximas dos valores-limite, as directivas sobre o meio ambiente na Europa devem estimar as concentrações limites de poluentes do ar tendo em consideração os efeitos sinérgicos entre os constituintes do aerossol, para melhor protecção da saúde de indivíduos mais sensíveis a alergias respiratórias, e para boa fertilidade de plantas espermatófitas. São ainda necessárias várias pesquisas sobre efeitos sinergéticos entre biopoluentes e substâncias químicas atmosféricas, na busca da causa do aumento de prevalência de alergias respiratórias na Europa.

Sugere-se que estes resultados sirvam de base para elaboração de um sistema de alerta auxiliar à prática médica (diagnóstico e aplicação de imunoterapia adequada), bem como para a qualidade de vida dos doentes, permitindo o planeamento das atividades diárias, como é o caso das que ocorrem ao ar livre.

Glossário

Glossário

Aerobiologia: Ciência que estuda as partículas biológicas presentes na atmosfera, sua dispersão e deposição, bem como o seu impacto no meio ambiente e nos organismos.

Aerossol: Material de dimensões muito reduzidas, disperso na atmosfera ou em qualquer outro ambiente gasoso.

Alergénio: É uma glicoproteína, que após contacto, é capaz de induzir sintomas de doenças alérgicas em indivíduos previamente sensibilizados. O antigénio é reconhecido pelo sistema imunitário que, subsequentemente, desenvolve um anticorpo específico ou imunidade celular. Os alergénios mais frequentes são os de grãos de pólen, ácaros, esporos fúngicos, caspa de cão e gato, alimentos e medicamentos.

Alergia: Resposta anormal e específica do sistema imunológico a uma substância estranha ao organismo, ou seja, uma hipersensibilidade imunológica a um estímulo externo específico.

Anticorpo ou Imunoglobulina: É uma proteína sintetizada e excretada por células plasmáticas derivadas dos linfócitos-B presentes no plasma. São tecidos e secreções que atacam proteínas estranhas ao corpo (denominadas antigénios), realizando assim a defesa do organismo (imunidade humoral). Depois de o sistema imunológico entrar em contacto com um antigénio, são produzidos anticorpos específicos contra ele. O anticorpo envolvido em reações alérgicas em seres humanos é o IgE.

Antigénio: Partícula ou molécula capaz de desencadear uma resposta imune, iniciada através do reconhecimento pelos linfócitos e que termine com a produção de um anticorpo específico.

Atopia: Distúrbios orgânicos de hipersensibilidade a diversos fatores ambientais, geralmente de carácter genético, porém, nem sempre são hereditários. As doenças atópicas mais frequentes são a asma brônquica, rinite alérgica, conjuntivite alérgica, febre dos fenos, síndrome da hipereosinofilia e alergias alimentares. A maioria dos indivíduos com atopia apresenta hiperprodução de IgE.

Atmosfera: Camada gasosa que envolve a superfície da Terra. É composta por azoto (78,08%), oxigénio (20,95%), árgon (0,93%), dióxido de carbono (0,038%), hélio (0,0018%), ozono (0,00006%), hidrogénio (0,00005%), vapor de água (quantidades variáveis, em média 1%) e gases raros (néon, xénon, crípton e hélio, em quantidades vestigiais). As camadas atmosféricas são: Troposfera, Estratosfera, Mesosfera, Termosfera, e exosfera. Estas são distintas e separadas entre si por áreas fronteiriças de descontinuidade (Tropopausa, Estratopausa, Mesopausa, Termopausa e Exobase).

Bioaerossol: Aerossol constituído por partículas de origem ou atividade biológica, dispersas no ar. As dimensões destas partículas podem oscilar entre 0,5 e 100 µm.

Citocinas: Termo genérico utilizado para designar um extenso grupo de moléculas envolvidas na emissão de sinais entre células, durante o desencadeamento das respostas imunes. Constituem um grupo de fatores extra-celulares que podem ser produzidos por diversas células, como monócitos, macrófagos, linfócitos e outras que não sejam linfóides. Todas citocinas são pequenas proteínas ou peptídeos, algumas contêm moléculas de açúcar ligadas (glicoproteínas).

Fumigação do Pólen: Exposição *in vitro* do pólen a poluentes numa câmara.

Germinação do pólen: Pólen germinado, é aquele que apresenta comprimento do tubo polínico maior em relação ao diâmetro do seu grão.

Hipótese nula: Hipótese que é presumida verdadeira até que provas estatísticas, sob a forma de testes de hipóteses, indiquem o contrário. O teste é baseado na observação de uma amostra aleatória da população.

Histamina: Mediador das respostas alérgicas na pele, no nariz e nos olhos, causando vasodilatação, aumento da permeabilidade vascular (edema) e contracção dos músculos lisos.

Humidade Relativa: quantidade de vapor de água que existe numa porção da atmosfera em relação a quantidade máxima de vapor de água que a atmosfera pode suportar a uma determinada temperatura. É geralmente expressa em percentagem (%).

Immunoblotting: Método imunológico para isolar e medir quantitativamente substâncias imunoreativas, ou seja, é um método para identificação de antigénios. Quando usado com reagentes imunes, tais como anticorpos monoclonais, o processo é genericamente conhecido como análise de Western blot. Nesta técnica, os antigénios separados por electroforese são deixados aderir às membranas de nitrocelulose e são identificados pela sua reação com anticorpos marcados. O método é também usado para detetar proteínas monoclonais.

Immunoblot: Resultado de análise ou identificação de proteínas específicas por reacções de antigénio-anticorpo.

Imunoglobulina E (IgE): classe de anticorpos (ou isotipo de imunoglobulina) que desempenha um papel importante em reacções alérgicas no organismo, e está, especialmente, associada a resposta hipersensitiva de tipo I. A IgE é uma imunoglobulina menos abundante em pessoas saudáveis, contudo, em indivíduos atópicos encontra-se geralmente em níveis elevados.

Imunologia: Estudo do sistema imunitário, especialmente, os aspetos fisiológicos, bom ou mau funcionamento de imunidade como: doenças auto-

imunes, hipersensibilidade, deficiência imune, rejeição pós-transplante, características físicas, químicas e fisiológicas dos componentes do sistema imunitário.

Indivíduos Atópicos: Pessoas com uma predisposição genética para desenvolverem doenças alérgicas.

Patogénico: Organismo capaz de induzir doença em outro ser vivo.

Pólen: Gametófito masculino produzido nas anteras das plantas superiores, cujo principal papel, é intervir na reprodução sexual de espermatófitas.

Poluentes Atmosféricos: São substâncias químicas de origem artificial ou natural, presentes na atmosfera (*geralmente no estado gasoso*), que podem atingir os seres vivos e causar efeitos deletérios.

Proteína: Composto orgânico constituído por uma cadeia de polipeptídeos. As proteínas desempenham várias funções no organismo dos seres vivos: catalisam reacções metabólicas, replicam ADN em resposta a estímulos e transportam moléculas de um local para outro. As proteínas são diferentes um do outro, principalmente na sua sequência de aminoácidos, que é ditado pela sequência de nucleótidos dos genes, e que geralmente resulta na dobragem da proteína para uma estrutura tridimensional específica que determina a sua actividade funcional.

Polinose: Reação de hipersensibilidade das vias respiratórias induzida por grãos de pólen, sendo caraterizada por manifestações alérgicas sazonais em indivíduos sensíveis, por afetar os pacientes durante a estação polínica.

Sensibilização alérgica: Produção de anticorpos do tipo IgE, em resultado da exposição de indivíduos sensibilizados a um alergénio.

Viabilidade do pólen: Capacidade do pólen para completar eventos de pós-polinização e alcançar a fertilização.