

Evaluation of the Effect of Pollution and Fungal Disease on *Pinus radiata* Pollen Allergenicity

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Key Words

Allergenicity • Allergic diseases • Ozone • Pine pollen • Pollution

Abstract

Background: Pollutants and other stressing factors like mold infection might increase the production of pathogen-related proteins in plants. Since this is invoked as one of the causes for the high prevalence of allergic diseases in developed countries, we aimed to determine the potential effect of environmental pollution, with or without mold infection of the trees, on the allergenic potency of pine pollen (*Pinus radiata*). **Methods:** Pine pollen samples were recovered from three selected areas: low polluted (A), highly polluted (B) and highly polluted and infected with fungi (*Sphaeropsis sapinea*) (C). The allergenic potency of pollen from areas A, B or C were compared in vivo in 35 pine pollen-allergic patients by skin prick test and specific IgE (sIgE) quantification. Pollen was also analyzed in vitro by SDS-PAGE immunoblotting, RAST inhibition and cDNA-AFLP (amplified fragment length polymorphism) to compare differences in proteins and mRNA expression. **Results:** The allergenic potency measured by prick

test, sIgE and RAST inhibition was greater in pollen A, which was exposed to smaller amounts of NO_x, PM₁₀ and SO₂ but greater amounts of O₃. No differences were found in IgE-binding bands in immunoblotting or densitometry of the bands. In cDNA-AFLP, three homologous transcript-derived fragments were expressed in samples B only, with an expressed sequence tag related with stress-regulated gene expression. **Conclusions:** A greater allergenic potency, in terms of skin tests and sIgE, is observed in pine pollen coming from unpolluted areas. We consider that this fact might be related to a higher exposure to ozone, resulting in a greater expression of allergenic proteins.

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Introduction

In the past, pollen from pine trees and other conifers was considered as poorly allergenic, but in recent years such consideration has been modified. Examples include cases of Cupressaceae pollen in France [1] and Italy [2], and *Cryptomeria japonica* in Japan [3].

Worldwide evidence suggests that allergic respiratory diseases have become more common in recent years [4]. Besides, these diseases appear to be more prevalent in industrialized compared with less industrial countries, and the incidence seems to be higher in polluted areas [5], especially areas with heavy traffic.

Effects of air pollution on increasing respiratory allergy have been attributed to: (1) the interaction of air pollutants with pollen grains, leading to an increased release of antigens; (2) paucimicronic particles (PM_{2.5-10}) carrying allergens with the inhaled air into the peripheral airways, thus inducing asthma in sensitized subjects; (3) an inflammatory effect of ozone, PM_{2.5-10} and sulfur dioxide on the airways, increasing the permeability of the mucous membrane for pollen allergens, and (4) an adjuvant immunologic effect of diesel exhaust particles on IgE synthesis in atopic subjects [6, 7].

Behrendt et al. [8] described that pollen collected from trees near roads with heavy traffic had particulate matter adhered to them, whereas pollen from park trees had not. Moreover, in vitro exposure of pollen grains to particles has been shown to modify the morphologic structure of the pollen, which might induce local allergen release [9]. Other studies on pollen from polluted areas have shown an increased production of allergens belonging to pathogenesis-related-proteins from plants. Armentia et al. [10] described an increase in the allergenicity of grass pollen collected in an urban area, apparently linked to an increased expression of Lol p 5. This effect was also described for Cupresaceae pollen, with its major allergen Cup a 3 being expressed only in cypress pollen obtained from an area polluted by heavy traffic [11].

Pollen from *Pinus radiata* (PR), an American pine belonging to the Pinaceae family of conifers formerly considered as non-allergenic, has also recently been reported as a cause for respiratory allergy in the Basque Country, a highly industrialized area in Northern Spain [12]. The presence of PR in the Basque Country is extensive: about 42% of all the forest areas (approximately 25% of the total Basque Country) are covered by managed plantations of this tree, since PR wood is widely exploited in the area for paper manufacturing and other commercial purposes.

Allergy to pine pollen was an infrequent cause of pollinosis in the area of the study, but its incidence might increase [12]. Nine different allergenic bands were described by the same authors, drawing attention to two main allergens with an apparent molecular weight of 42 and 6–8 kDa, respectively.

Hypothetically, the allergenicity of PR pollen in the Basque Country could be increased by two factors: (1) the

neighborhood of some tree plantations to polluted areas, and (2) the widespread infection by *Spheropsis sapinea*, a conifer fungal pathogen that causes shoot blight and stem cankers. Latent infections with *S. sapinea* are common and cause significant mortality in pines; although the precise mechanism of the disease is unknown, it is hypothesized that the production of phytotoxins causes stress and modifies in some way the characteristics of pollen.

The aim of the present study was to evaluate the effect of pollution and fungal infections by *S. sapinea* on the allergenic potency of PR pollen.

Patients and Methods

Selection of PR Plantations

Different pine plantations were selected according to the level of air pollution and the presence of fungal infection. Sample A was collected in a non-polluted rural area from non-infected trees; sample B in a highly polluted area close to heavy traffic roads from non-infected trees, and sample C was collected in an equally polluted area from trees infected with *S. sapinea*.

The exposure to different pollutants (NO, NO₂, NO_x, SO₂, O₃ and PM₁₀) was continuously measured in each study area during the 2-year study period by means of a standard automatic monitoring station (Department of Environmental Control, Basque Country Government). The characteristics of the different areas and levels of contaminants are detailed in table 1. Trained biologists with proven expertise in this disease determined whether or not a tree was healthy or infected by the fungus *S. sapinea*.

To avoid the influence of other confounding factors such as soil quality, climate conditions, rain fall or temperature, all three sampling areas were within an area of 50 km², forming a triangle of 20 km per side, and separated by mountains (for a precise location, see table 1).

Pollen Collection and Extraction

Samples of pollen were collected from 5 trees in each of the three study areas during the peak pollinization period (February and March) in 2 different years (2006 and 2007). Catkins were collected at a slightly earlier stage of pollen development, i.e. after they had turned yellow and produced very little liquid when squeezed between the fingers. Then, they were placed in paper bags, stored for 2 days at 21–25°C. Pollen was extracted in warm and dry atmosphere (27°C and 20–40% relative humidity). The spikes were shaken and sieved, and their pollen was collected, stored and kept refrigerated at 4°C.

For the allergen extraction, pollen humidity from each sample was first measured using the Karl Fisher method [13]. Then, 6 g of each type of pollen were selected to elaborate the extracts. Protein extraction was performed by stirring at 10% (w/v) in phosphate-buffered saline (PBS) for 90 min at 4°C. Samples were centrifuged at 12,500 rpm for 30 min and the recovered supernatants were dialyzed against distilled water. The dialyzed material was filtered (0.45- and 0.22-μm filters) and stored at –20°C. The amount of proteins was measured by the Lowry method [14]. Subsequently, extracts were adjusted to the same protein concentration (1 mg/ml).

Patients

Thirty-five pine pollen-allergic patients (14 males and 21 females with a mean age of 48 years) were recruited from patients attending one of four hospitals in the Basque Country. All of them had rhinoconjunctivitis and 40% had asthma. The diagnosis of allergy to pine pollen was suspected by the presence of symptoms during the pine pollen season and confirmed by a positive skin prick test. Eleven patients were monosensitized to PR. Prior to the enrolment, the study protocol was approved by the corresponding ethics committee and all patients provided written informed consent.

Skin Prick Test

To evaluate the allergenic potency of the pollen extracts to the assay, a skin prick test was performed to all patients outside the pine pollen season using four diluted concentrations (1/1, 1/5, 1/25 and 1/125) of each original extract (A, B and C) from pollen collected in 2006. In brief, skin tests were performed on the volar forearm (between 5 cm above the wrist and 3 cm below the elbow). Positive (10 mg/ml histamine) and negative (saline solution) controls were also tested. The area of each wheal was measured by planimetry, and the results were subsequently analyzed by means of the parallel line assay [15].

Specific IgE Determinations

Specific IgE (sIgE) against (1) study pollen extract and (2) commercially available extract was assessed in all patients.

The quantification of IgE against the collected samples (A, B and C) was performed by the technique described by Sander et al. [16]. Briefly, protein extracts were biotinylated using a commercially available biotin protein labeling kit (Roche, Paris, France). The labeled pollen proteins were then bound to streptavidin ImmunoCAP (Phadia, Uppsala, Sweden), and then applied to a Phadia UniCAP® system with patient sera.

In addition, sIgE determinations against commercially available pine pollen, both PR (t213) and *Pinus strobus* (PS; t16), were also performed using the UniCAP system [17]. In both cases (raw and commercial extracts), values >0.35 kU/l were considered positive.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting were performed in each individual patient (data not shown) and additionally in a pool of 10 selected patients.

SDS-PAGE was carried out according to the method of Laemmli. In brief, equal amounts of protein A, B and C extracts, and a commercial extract of PR (ALK-Abelló, Madrid, Spain) were precipitated with 5% phosphotungstic acid, and after resuspension, they were loaded onto 10–20% Tris-glycine gels (Novex, San Diego, Calif., USA), separated under reducing conditions and silver stained (Silver Stain Plus, Bio-Rad Laboratories, Hercules, Calif., USA). A negative control without serum was also included.

Electrophoretic bands were transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories), essentially as described by Towbin et al. [18]. For immunoblot analysis, the membranes were blocked with 5% bovine serum albumin solution in PBS. Strips were incubated overnight with serum samples diluted 1:5. The membranes were then incubated with a mouse monoclonal antibody to human IgE (HE-2) [19] and subsequently with a rabbit anti-mouse antibody conjugated with horseradish peroxi-

dase. Then, the membranes were treated with the ECL chemiluminescent substrate (Amersham ECL-Plus Western blotting detection system, GE Healthcare UK Ltd, Little Chalfont, UK) and the reactive bands were identified by means of exposure to X-ray film (Kodak S-OMAT; Sigma, St. Louis, Mo., USA). Finally, the software Quantity One (Bio-Rad) was utilized to calculate the density of the resulting bands.

In vitro Quantitation of the Biological Activity of the Extracts

The biological activity of each extract was determined by RAST inhibition and expressed as the quantity of extract required for 50% inhibition of IgE binding.

Paper discs were activated with cyanogen bromide and subsequently conjugated with the PR extract according to the method of Ceska et al. [20]. The pine discs were incubated in duplicate with 50 µl of a pool of patient sera (1:5 dilution) and 50 µl of inhibiting extract (A, B and C) at different protein concentrations, and then with 100,000 cpm of ¹²⁵I-anti-human IgE mAb HE-2 [18]. The biological activity of extracts A, B and C [expressed in HEP (histamine equivalent potency units)] was determined by comparison of the inhibition curves obtained with the reference extract curve previously calibrated.

Analysis of cDNA-AFLP

To study the differences in mRNA expression between two areas with different degrees of pollution, bud samples were collected during the time of flower induction (October) from four trees selected in non-polluted (A1, A2, A3 and A4) and polluted areas (B1, B2, B3 and B4) and immediately stored at –80°C. In addition, mixes of different pollens were also assayed in the same gel (MA and MB).

Total RNA was isolated from about 5 g of frozen tissue following the procedure of Chang and Cairney [21], with some modifications [22]. Therefore, total RNA concentrations were estimated by spectrophotometry and visualized on 1% agarose gels. Poly-A RNA was obtained from 50 µg of total RNA using 5'-biotinylated oligo(dT) primer bound to paramagnetic beads coated with streptavidin (Dyanabeads M-280 streptavidin, DYNAL, Oslo, Norway). First- and second-strand cDNAs were synthesized according to Sambrook [23]. Double-strand cDNA (ds-cDNA) was digested with *AseI* and *TaqI* (NEB Biolabs, New Brunswick, Nebr., USA) followed by ligation of *AseI* and *TaqI* adapters with T4 DNA ligase (Invitrogen, Barcelona, Spain). PCR for cDNA-AFLP (amplified fragment length polymorphism) was carried out as described by Bachem and Visser [24]. Primers were labeled for the specific amplifications with either fluorescent infrared dyes IRD800 or IRD700 (LI-COR, Lincoln, Nebr., USA). Amplification products were denatured and separated on 6% denaturing polyacrylamide (19:1) gels. They were visualized on a DNA sequencing and fragment analysis system (LI-COR 4200-S1) as described by the manufacturer (LI-COR, 1997).

Polymorphic bands (based on their presence/absence) were excised from the polyacrylamide gel, rehydrated and eluted after purification. DNA was reamplified using the same primer combination as for selective amplification. Sequence determination was carried out using an automated sequencer AB3130 (Applied Biosystems). Sequence homology searches were performed in the GenBank (<http://www.ncbi.nlm.nih.gov/>) using the BLASTn and BLASTx search algorithms [25].

Table 1. Characteristics of the samples studied and level of contaminants in each area (means of daily data from 2 years)

Sample	Location	Trees	Mean concentrations (95% CI) of pollutants (2006–2007), $\mu\text{g}/\text{m}^3$					
			NO_2	NO	NO_x	O_3	PM_{10}	SO_2
A	rural 43°02'19.34"N/2°56'48.46"O	healthy	21.1 (20.5–21.7)	10.6 (9.8–11.4)	37.4 (35.7–39.1)	45.9 (44.5–47.4)	24.9 (24.9–25.9)	6.4 (6.3–6.6)
B	near road 43°11'29.92"N/2°53'43.15"O	healthy	28.9 (28.6–29.1)	19.7 (18.4–20.9)	60.4 (57.9–62.8)	32.0 (30.7–33.2)	28.8 (27.7–30.0)	7.6 (7.4–8)
C	near road 43°03'15.37"N/2°55'08.44"O	infected by <i>S. sapinea</i>	35.9 (35.1–36.7)	21.7 (20.4–23.0)	68.5 (65.9–71.0)	36.2 (34.8–37.5)	40.1 (38.4–41.9)	7.3 (7.2–7.5)

Statistical Analysis

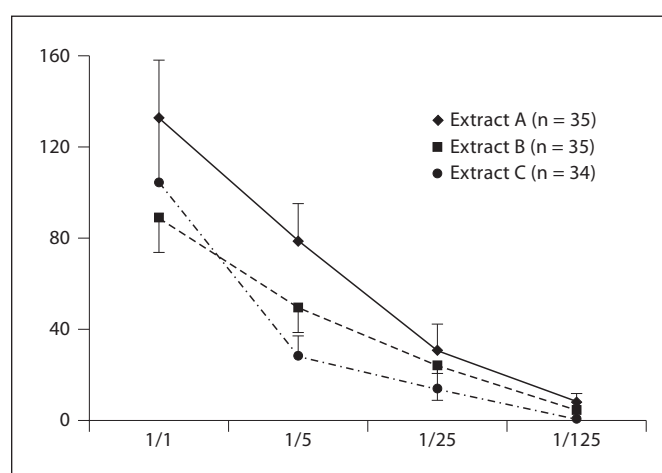
Results are described as means and 95% confidence intervals (CI). Variations in cutaneous reactivity to different extracts determined by skin prick test were analyzed using the parallel line assay by means of a specific ALASA CRS PLA software [26–28], based on the parallelism of regression lines calculated with the increasing concentrations of each extract assayed. Comparison of the biological activity between extracts was performed by the relative comparison with the extract showing the higher potency and expressed as 95% CI. Differences in IgE levels between extracts A, B and C were evaluated by means of a general linear model of analysis of variance for repeated measures, followed by post hoc analysis when appropriate. The relationship of IgE levels with the different extracts was analyzed by means of Pearson's correlation. The value of p is quoted whenever significant ($p < 0.05$).

Results

Figure 1 shows the mean wheal sizes obtained by prick test for each dilution of the extracts. Extract A showed the highest allergenic potency, with a higher geometric mean of the wheal for all dilutions, and was therefore considered as 100%. In comparison with A, the relative potency of extract B was 84.4% (95% CI: 66.7–106.8; nonsignificant) and the potency of extract C was 62% (95% CI: 44.2–87.2; $p < 0.01$). In addition, extract C was also significantly less potent than B (73.3%; 95% CI: 57.5–93.3; $p < 0.05$).

Results of sIgE are detailed in table 2. Comparing non-commercial extracts, the highest IgE mean level was obtained with extract A ($p < 0.01$ vs. B). No significant differences were found between extracts A and C or between B and C. Comparing commercial CAPs, no statistical differences were observed in the mean levels of sIgE between PR and PS.

A strong correlation in IgE levels was noted between the three experimental extracts (A vs. B: $r = 0.993$; $p <$

**Fig. 1.** Mean of the wheal areas obtained with the four different dilutions of extracts A, B and C from PR pollen.

0.001; A vs. C: $r = 0.978$, $p < 0.001$, and B vs. C: $r = 0.993$, $p < 0.001$) and also between the two commercial extracts (PS vs. PR: $r = 0.959$, $p < 0.001$). In contrast, a poor correlation was observed between the non-commercial and commercial extracts, either against PR (A vs. PR: $r = -0.03$; B vs. PR: $r = -0.17$; C vs. PR: $r = -0.065$) or PS (A vs. PS: $r = -0.221$; B vs. PS: $r = -0.217$; C vs. PS: $r = -0.176$).

Immunoblotting of the commercial and natural extracts showed a similar pattern, with four IgE-binding bands with an apparent molecular weight (MW) of 6–8 and 40 kDa, and a group of two bands of 55–140 kDa (fig. 2). The extracts did not significantly differ in the mean density or the percentage of patients with an apparent band. None of the extracts had a specific allergenic band.

Table 2. Results of sIgE determinations (expressed in kU/l) and allergens recognized in immunoblotting in all the patients

Patient No.	Extract A	Extract B	Extract C	PR (t213)	PS (t16)	6–8 kDa band	40 kDa band	>55 kDa band
1	6.2	5.4	6.6	1.6	1.73	X	X	X
2	0.8	0.6	0.7	0.35	0.27		X	X
3	40.3	23.4	20.6	2.7	2.86		X	X
4	100.0	87.4	100.0	1.5	8.26	X		
5	2.2	1.9	1.7	1.4	1.47		X	X
6	42.3	37.7	47.6	0.7	7.18	X		
7	0.4	0.2	0.2	6.1	5.43			
8	22.1	16.9	18.5	1.0	2.36	X	X	X
9	0.0	0.0	0.0	0.0	0.04			
10	1.3	1.5	0.9	0.1	0.14		X	X
11	23.2	17.6	16.6	10.7	9.26	X	X	X
12	4.6	3.7	4.4	0.2	0.71	X		
13	22.3	19.2	24.8	0.6	5.82		X	
14	3.9	3.1	3.9	0.1	0.09	X		
15	0.4	0.2	0.1	8.8	8.54	X	X	
16	4.2	3.0	3.8	23.6	18.7			X
17	26.4	19.4	24.4	0.5	0.51	X	X	X
18	1.4	0.7	0.7	13.0	9.91			
19	5.4	4.1	4.9	0.1	0.46	X		X
20	0.6	0.5	0.5	1.6	1.31		X	
21	1.1	0.7	0.7	0.1	0.18			X
22	5.2	2.9	2.6	0.2	0.47		X	X
23	0.2	0.1	0.0	0.0	0.02			
24	1.4	0.5	0.4	14.4	9.72	X		
25	8.5	6.9	4.6	30.4	23.5		X	
26	0.9	0.7	0.6	0.1	0.1		X	X
27	1.1	0.8	1.1	0.2	0.17		X	X
28	17.7	12.7	10.5	28.6	24.6		X	X
29	1.1	0.6	0.6	0.0	0.11		X	X
30	5.7	3.8	5.2	0.0	0.19	X		
31	0.8	0.5	0.6	0.0	0.07			
32	1.7	1.3	1.0	0.2	0.42		X	
33	0.9	0.7	0.9	0.0	0.07	X	X	
34	0.0	0.0	0.0	2.3	1.4	X		X
35	0.0	0.0	6.4	0.0	0.04	X	X	
Mean	10.1	8.0	9.0	4.4	4.2			
L 95% CI	3.7	2.6	2.8	1.6	2.0			
H 95% CI	16.5	13.4	15.3	7.2	6.3			

X = This allergen is recognized by the patient serum; L 95% CI = low 95% confidence interval; H 95% CI = high 95% confidence interval.

Patients which recognized the lowest MW band (6–8 kDa) in all natural extracts had higher IgE levels against PR than those who did not, but these differences were not statistically significant. However, when sIgE was measured against commercially available t213 (PR) or t16 pollen (PS), levels were higher in patients who did not recognize the 6- to 8-kDa band (table 3).

Analysis of the biological activity by RAST inhibition showed higher allergen activity of the extract obtained in 2006 from non-polluted (A) in comparison to polluted areas (B) and the extract of pollen obtained from trees infected with the fungus in a polluted area (C). In 2007, the results of allergen activity were also higher in extract A in comparison to B. However, during this year, allergen activity of extract C was the highest (table 4).

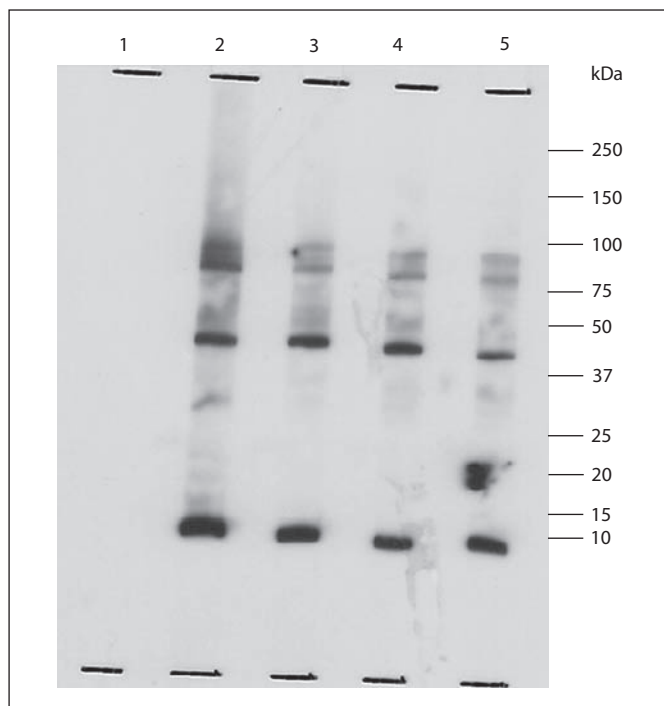


Fig. 2. Immunodetection in the extracts of PR pollen collected in 2006 using a pool of 10 selected sera. Lane 1 = Negative control; lane 2 = commercial PR extract (ALK-Abelló); lane 3 = extract A; lane 4 = extract B; lane 5 = extract C.

Table 3. Mean sIgE to the different extracts in patients positive/negative to the 6- to 8-kDa allergen

Patients	IgE, kU/l				
	t213	t16	A	B	C
6–8 kDa negative (n = 20)	5.4	4.8	5.6	3.9	3.8
6–8 kDa positive (n = 15)	2.8	3.4	16.2	13.4	15.9

The differences were not statistically significant.

Table 4. Allergen potency of the different extracts in both years

Extract	2006	2007
Pollen A	122	70
Pollen B	65	39
Pollen C	92	74

The results are expressed in HEP/g of dried pollen.

Using 10 primer combinations, about 120 cDNA fragments were counted, but only three of them (transcript-derived fragments) were expressed in the samples from polluted areas but not in extract A (fig. 3). These fragments were compared with those present in the GenBank database (table 5) and all the three fragments showed almost perfect homology with EST related with stress-regulated gene expression, suggesting that those fragments were induced by standard contamination.

Discussion

Environmental pollution is one of the factors involved in the increased incidence of allergic diseases in the last decades. It has been suggested that pollution might produce irritation and also inflammation of the airways; furthermore, the particles could act as allergen transporters to small airways [6].

Theoretically, pollution could also affect the sources of allergens. Plants exposed to urban or traffic pollution could suffer stress with the resultant increase in the production of pathogenesis-related proteins (PRP) [29, 30]. A higher expression of a defense protein, or even the expression of a new one, could enhance the allergenic potency of the pollen. The PRP 2, 3, 4, 5, 8, 10 and 14 groups contain allergenic proteins. Examples include Bet v 1 (birch), Cor a 1 (hazel), Mal d 1 (apple), Api g 1 (celery) and Phl p 5 (*Phleum pratense*). One study found an increase in the allergenic strength of pollen collected in an area exposed to traffic fumes, and the expression of an allergen (Cup a 3) only in cypress pollen exposed to pollution [11]. Another similar study detected an increase in the allergenicity of pollen from *Lolium perenne* collected in an urban area in comparison to that collected in a rural one [10]. Unfortunately, neither of these studies provides data on the levels of air pollutants in the pollen collection areas. In a study performed on guinea pigs with *Zinnia* pollen from urban and rural areas, the size of the skin tests and IgE levels were greater with the polluted pollen, but no blotting differences were found in IgE-binding bands [31].

Hypothetically, the presence of atypical molecules in pollen, e.g. during fungal infestation, could also be another modulator of allergenic responses, either directly – as a result of stress – or via the expression of toxins [32].

In accordance with all the above-mentioned knowledge, we formulated the initial hypothesis of a higher allergenicity of pine pollen from polluted areas. Sur-

prisingly, in our study, the pollen from the least polluted area confirmed to be the most allergenic by several approaches.

Expected differences in pollutants between areas were confirmed, so the areas selected as polluted (B and C) had higher levels of NO, CO, SO₂ and PM_{2.5-10} compared to the non-polluted area (A). In contrast, ozone levels were higher in area A, as expected. Pollen sample C was obtained from trees infected with a fungal disease which is highly prevalent in the studied region. The differences between areas were also as expected for cDNA-AFLP analysis, which identified 3 stress-related genes differentially up-regulated in the contaminated areas, but not in the unpolluted one.

However, contrary to our initial hypothesis based on results from in vivo studies, skin prick test or sIgE quantification clearly showed that the extract from the unpolluted area (A) was the most allergenic. These results were further corroborated in vitro with the RAST inhibition study, which also showed a higher allergenicity of extract A. Also contrary to the initial hypothesis, the effect of stress due to pollutants or fungal infection on the trees was not evident in the pollen, since the allergenic bands detected by immunoblotting were the same in all three extracts.

Differences in sIgE levels to PR by commercial CAP or ImmunoCAP with respect to the three study extracts (A, B and C) could also be explained by the greater exposure to ozone. Unfortunately, the level of exposure to contaminants of the pollen used in commercial ImmunoCAP was unknown. According to data provided by the laboratory, ImmunoCAP for PR is made up of a collection of

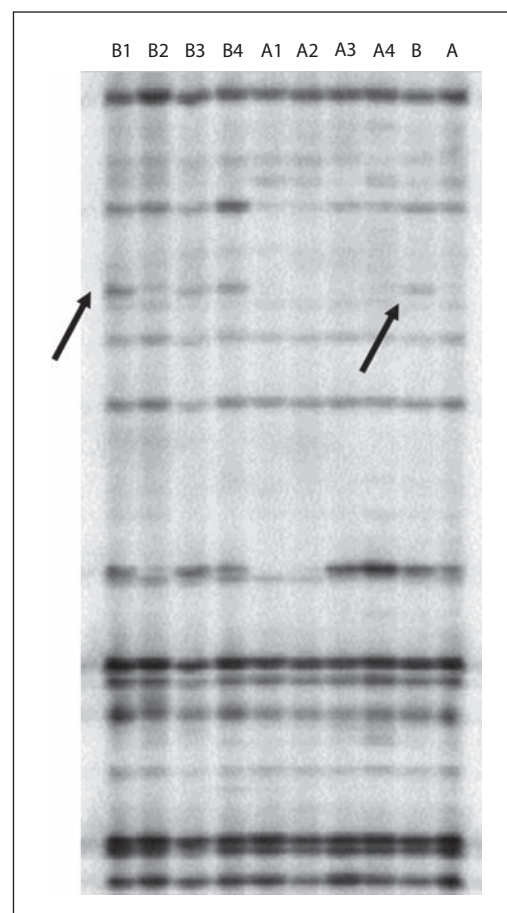


Fig. 3. Result of cDNA-AFLP. In pines located in a polluted area (B1, B2, B3, B4), a common band (fragment p115) can be noted (arrows) that does not appear in the pines from a non-polluted area (A1, A2, A3 and A4). Lanes B and A are a mix of cDNA from trees located in polluted and non-polluted areas, respectively.

Table 5. Transcript-derived fragments of mRNA detected only in polluted samples and their homology with other fragments from GenBank

TDF	Length bp	Accession No.	Homology	BLAST score
DEPR1	115	DR118989	roots minus magnesium <i>Pinus taeda</i> cDNA clone RTMG1_20_A10_A029 5', mRNA sequence	8e-53
DEPR1	115	DR093085	stem response resistant <i>Pinus taeda</i> cDNA clone STRR1_66_A01_A033 3', mRNA sequence	8e-53
DEPR1	115	CF391924	drought-stressed loblolly pine roots DS2 <i>Pinus taeda</i> cDNA clone RTDS2_1_H12_A021 3', mRNA sequence	4e-51
DEPR2	538	CO174776	needles control <i>Pinus taeda</i> cDNA clone NDL1_46_E10_A029 3', mRNA sequence	0
DEPR3	569	DR100636	stem response resistant <i>Pinus taeda</i> cDNA clone STRR1_66_C05_A033 3', mRNA sequence	0
DEPR3	569	DR054034	roots minus calcium <i>Pinus taeda</i> cDNA clone RTCA1_14_G06_A029 5', mRNA sequence	0
DEPR3	569	DR013599	root at 37° C for 24 h <i>Pinus taeda</i> cDNA clone HEAT1_20_G04_A029 3', mRNA sequence	8e-98
DEPR3	569	DR384663	roots plus added mercury <i>Pinus taeda</i> cDNA clone RTHG1_3_E10_A029 5', mRNA sequence	3e-97

pollens from different locations in Europe (e.g. Spain, Portugal and Croatia) and is composed of 5 proteins (82, 67, 54, 44 and 38 kDa). However, in our study, the patients that recognized the 6- to 8-kDa allergen (not included in the commercial IgE quantification) had higher sIgE levels against PR with the natural extracts but lower sIgE to PR with the commercial technique. Consistent with its molecular weight, this 6- to 8-kDa allergen could correspond to a polcalcin (ubiquitous Ca^{2+} -binding proteins present in pollens and considered as panallergens), which can be modulated by stress. The remarkable differences found between the sIgE levels determined with the commercial CAP and extracts A, B and C suggest that the source of the products used in the manufacture of allergenic extracts should be taken into account. Another factor to be considered is the impact of the manufacturer on the potency or allergenicity of a given extract. It has previously been demonstrated that commercial extracts with the same composition and the same nominal dose but manufactured by different laboratories have relevant differences in systemic reactions, resulting in some cases in a significant increase in patient risk [33]. Probably, it would be advisable to study the variability in allergenic potency according to the source of the raw material used in the production of other allergenic extracts. In the future, such problems might definitively be solved by the use of purified or recombinant allergens for diagnosis and treatment.

As stated, a higher ozone concentration was detected in the uncontaminated area (A). Ozone is produced in the presence of sunlight and requires precursor pollutants such as nitrogen oxides and volatile organic compounds from vehicle engines. It is a well-known fact that ozone levels are higher in rural locations close to urban areas [34, 35]. Several studies demonstrated the harmful effects of ozone on human health [36–39]; apart from the direct effect on the airways described in these studies, ozone may act by other mechanisms. Motta et al. [40] found an increase in the harmful effects of pollen from *Phleum* together with an increase in the release of cytoplasmic granules following exposure to NO_2 and O_3 . Protein nitration has been observed in allergens such as Bet v 1 exposed to NO_2 and O_3 [41]. Besides, protein nitration increases the allergenic potency of ovalbumin or Bet v 1 [42].

Ozone has also an intense oxidizing effect and a great capacity for inducing stress in plants. Ozone levels reaching $100 \mu\text{g}/\text{m}^3$ every 8 h are considered harmful for plants. Effects that have been described include changes in color and appearance of leaves and shoots, physiologic

alterations such as a reduction in CO_2 elimination, pore conductance and transpiration, and changes in the ability to perform photosynthesis and amounts of chlorophyll [43–45]. Exposure of plants to ozone has also been shown to induce defense genes against pathogens [46–48] although an association of these proteins with an increase in allergenicity has not been reported.

A recent controlled experiment demonstrated that the exposure of a rye plantation to O_3 increased not only the content of proteins in pollen but also group 1, 5, 6 and profilin allergens of grasses in exposed rye. Immunoblotting in 2 grass-allergic patients also showed enhanced bands in O_3 -exposed pollen [49]. Another very recent prospective study including 1,430 children showed an association between the degree of ozone exposure with asthma and allergic rhinitis and an increased rate of newly developed sensitization to outdoor allergens [50].

In the case of pine pollen, ozone could have a stressing effect on the tree, which is even higher than that of other pollutants, which would lead to a greater allergenicity. However, the precise mechanism for O_3 to increase allergenicity remains unclear, since a greater production of certain allergens was not reflected in the IgE-binding band pattern from immunoblotting. The effect of ozone and other pollutants may perhaps have a cumulative effect in combination with other stimuli such as climate stress or fungal infection by *S. sapinea*; therefore, further studies analyzing the independent effects of individual pollutants might be interesting. In addition, it must be borne in mind that previous studies have shown different effects based on whether the exposure to the pollutant took place in the laboratory or in the open air, and, also, that the response to stress varies from one plant species to another [43, 44, 51].

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References

- ▶ 1 Panzani R, Centanni G, Brunel M: Increase of respiratory allergy to the pollens of cypresses in the south of France. *Ann Allergy* 1986;56:460–463.
- ▶ 2 Iacovacci P, Afferni C, Barletta B, Tinghino R, Di Felice G, Pini C, et al: *Juniperus oxycedrus*: a new allergenic pollen from the Cupressaceae family. *J Allergy Clin Immunol* 1998;101:755–761.
- ▶ 3 Ishizaki T, Koizumi K, Ikemori R, Ishiyama Y, Kushibiki E: Studies of prevalence of Japanese cedar pollinosis among the residents in a densely cultivated area. *Ann Allergy* 1987;58:265–270.
- ▶ 4 Shamssain MH, Shamsian N: Prevalence and severity of asthma, rhinitis, and atopic eczema in 13- to 14-year-old schoolchildren from the northeast of England. *Ann Allergy Asthma Immunol* 2001;86:428–432.
- ▶ 5 Wuthrich B, Schindler C, Leuenberger P, Ackermann-Lieblich U: Prevalence of atopy and pollinosis in the adult population of Switzerland (SAPALDIA study). *Swiss Study on Air Pollution and Lung Diseases in Adults*. *Int Arch Allergy Immunol* 1995;106:149–156.
- ▶ 6 D'Amato G, Liccardi G, D'Amato M, Cazzola M: The role of outdoor air pollution and climatic changes on the rising trends in respiratory allergy. *Respir Med* 2001;95:606–611.
- ▶ 7 D'Amato G, Liccardi G, D'Amato M, Holgate S: Environmental risk factors and allergic bronchial asthma. *Clin Exp Allergy* 2005;35:1113–1124.
- ▶ 8 Behrendt H, Becker WM, Friederichs KH, Darsow U, Tomingas R: Interaction between aeroallergens and airborne particulate matter. *Int Arch Allergy Immunol* 1992;99:425–428.
- ▶ 9 Behrendt H, Becker WM, Fritzsche C, Sliwa-Tomczok W, Tomczok J, Friederichs KH: Air pollution and allergy: experimental studies on modulation of allergen release from pollen by air pollutants. *Int Arch Allergy Immunol* 1997;113:69–74.
- ▶ 10 Armentia A, Lombardero M, Callejo A, Barber D, Martin Gil FJ, Martin-Santos JM, et al: Is *Lolium* pollen from an urban environment more allergenic than rural pollen? *Allergol Immunopathol (Madr)* 2002;30:218–224.
- ▶ 11 Cortegano I, Civantos E, Aceituno E, del Moral A, Lopez E, Lombardero M, et al: Cloning and expression of a major allergen from *Cupressus arizonica* pollen, Cup a 3, a PR-5 protein expressed under polluted environment. *Allergy* 2004;59:485–490.
- ▶ 12 Gastaminza G, Lombardero M, Bernaola G, Antepara I, Munoz D, Gamboa PM, et al: Allergenicity and cross-reactivity of pine pollen. *Clin Exp Allergy* 2009;39:1438–1446.
- ▶ 13 Verhoef JC, Barendrecht E: Mechanism and reaction rate of the Karl-Fischer titration reaction. *J Electroanal Chem* 1976;71:305–315.
- ▶ 14 Lowry OR, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–275.
- ▶ 15 Martin S, Cuesta P, Rico P, Cortes C: A computer program based on parallel line assay for analysis of skin tests. *Allergy* 1997;52:97–100.
- ▶ 16 Sander I, Kespohl S, Merget R, Goldscheid N, Degens PO, Bruning T, et al: A new method to bind allergens for the measurement of specific IgE antibodies. *Int Arch Allergy Immunol* 2005;136:39–44.
- ▶ 17 Paganelli R, Ansotegui IJ, Sastre J, Lange CE, Roovers MH, de Groot H, et al: Specific IgE antibodies in the diagnosis of atopic disease. Clinical evaluation of a new in vitro test system, UniCAP, in six European allergy clinics. *Allergy* 1998;53:763–768.
- ▶ 18 Towbin HS, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4354.
- ▶ 19 Sanchez-Madrid F, Morago G, Corbi AL, Carreira J: Monoclonal antibodies to three distinct epitopes on human IgE: their use for determination of allergen-specific IgE. *J Immunol Methods* 1984;73:367–378.
- ▶ 20 Ceska ME, Eriksson R, Varga JM: Radioimmunosorbent assay of allergens. *J Allergy Clin Immunol* 1972;49:1–9.
- ▶ 21 Chang SPJ, Cairney J: A simple method for isolating RNA from pine trees. *Plant Mol Biol Rep* 1993;11:113–116.
- ▶ 22 Azevedo H, Lino-Neto T, Tavares R: An improved method for high-quality RNA isolation from needles of adult maritime pine trees. *Plant Mol Biol Rep* 2003;21:333–338.
- ▶ 23 Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*. New York, Cold Spring Harbor Laboratory Press, 1989.
- ▶ 24 Bachem C, Oomen R, Visser R: Transcript imaging with cDNA-AFLP: a step by step protocol. *Plant Mol Biol Rep* 1998;16:157–173.
- ▶ 25 Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389–3402.
- ▶ 26 Martín S, Cuesta P, Rico P, Cortés C: A computer program based on parallel line assay for analysis of skin tests. *Allergy* 1997;52:97–100.
- ▶ 27 Finney D: *Statistical Method in Biological Assay*, ed 4. London, Griffin, 1978, pp 69–104.
- ▶ 28 Finney D, Schild HO: Parallel line assay with successive adjustment of doses. *Br J Pharmacol Chemother* 1966;28:84–92.
- ▶ 29 Stintzi AH, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Le-grand M, Fritting B: Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie* 1993;75:687–706.
- ▶ 30 Sels J, Mathys J, De Coninck BM, Cammue BP, De Bolle MF: Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiol Biochem* 2008;46:941–950.
- ▶ 31 Chehregani A, Majde A, Moin M, Gholami M, Ali Shariatzadeh M, Nassiri H: Increasing allergy potency of *Zinnia* pollen grains in polluted areas. *Ecotoxicol Environ Saf* 2004;58:267–272.
- ▶ 32 Evidente A, Fiore M, Bruno G, Sparapano L, Motta A: Chemical and biological characterisation of sapinopyridione, a phytotoxic 3,3,6-trisubstituted-2,4-pyridione produced by *Sphaeropsis sapinea*, a toxigenic pathogen of native and exotic conifers, and its derivatives. *Phytochemistry* 2006;67:1019–1028.
- ▶ 33 Gastaminza G, Algorta J, Audicana M, Etxenagusia M, Fernandez E, Muñoz D: Systemic reactions to immunotherapy: influence of composition and manufacturer. *Clin Exp Allergy* 2003;33:470–474.
- ▶ 34 Sanz MJ, Sanz F, Calatayud V, Sanchez-Pena G: Ozone in Spain's national parks and protected forests. *ScientificWorldJournal* 2007;7(suppl 1):67–77.
- ▶ 35 Mantilla EMM, Carratala A, Salvador R, Sanz MJ: Influencia de los procesos mesometeorológicos en la evolución de los niveles registrados de ozono en la Comunidad Valenciana I. *Proc Techn Workshop Ozone Pollut Southern Europe*. Valencia, 1997.
- ▶ 36 Wang JH, Devalia JL, Duddle JM, Hamilton SA, Davies RJ: Effect of six-hour exposure to nitrogen dioxide on early-phase nasal response to allergen challenge in patients with a history of seasonal allergic rhinitis. *J Allergy Clin Immunol* 1995;96:669–676.
- ▶ 37 Molino NA, Wright SC, Katz I, Tarlo S, Silverman F, McClean PA, et al: Effect of low concentrations of ozone on inhaled allergen responses in asthmatic subjects. *Lancet* 1991;338:199–203.
- ▶ 38 Gent JF, Triche EW, Holford TR, Belanger K, Bracken MB, Beckett WS, et al: Association of low-level ozone and fine particles with respiratory symptoms in children with asthma. *JAMA* 2003;290:1859–1867.
- ▶ 39 Takizawa H: Diesel exhaust particles and their effect on induced cytokine expression in human bronchial epithelial cells. *Curr Opin Allergy Clin Immunol* 2004;4:355–359.
- ▶ 40 Motta AC, Marliere M, Peltre G, Sterenberg PA, Lacroix G: Traffic-related air pollutants induce the release of allergen-containing cytoplasmic granules from grass pollen. *Int Arch Allergy Immunol* 2006;139:294–299.

- ▶ 41 Franze T, Waller MG, Niessner R, Pöschl U: Protein nitration by polluted air. *Environ Sci Technol* 2005;39:1673–1678.
- ▶ 42 Gruijthuijsen YG, Grieshuber I, Stöcklinger A, Tischler U, Fehrenbach T, Weller MG, Vogel L, Pöschl U, Duschl A: Nitration enhances the allergenic potential of proteins. *Int Arch Allergy Immunol* 2006;141:265–275.
- ▶ 43 Furlan CM, Moraes RM, Bulbovas P, Sanz MJ, Domingos M, Salatino A: *Tibouchina pulchra* (Cham.) Cogn., a native Atlantic Forest species, as a bio-indicator of ozone: visible injury. *Environ Pollut* 2008;152:361–365.
- ▶ 44 Moraes RM, Bulbovas P, Furlan CM, Domingos M, Meirelles ST, Delitti WB, et al: Physiological responses of saplings of *Caesalpinia echinata* Lam., a Brazilian tree species, under ozone fumigation. *Ecotoxicol Environ Saf* 2006;63:306–312.
- ▶ 45 Cano I, Calatayud V, Cervero J, Sanz MJ: Ozone effects on three *Sambucus* species. *Environ Monit Assess* 2007;128:83–91.
- ▶ 46 Sandermann H Jr: Ozone and plant health. *Annu Rev Phytopathol* 1996;34:347–366.
- ▶ 47 Marco F, Calvo E, Carrasco P, Sanz MJ: Analysis of molecular markers in three different tomato cultivars exposed to ozone stress. *Plant Cell Rep* 2008;27:197–207.
- ▶ 48 Savenstrand H, Brosche M, Strid A: Regulation of gene expression by low levels of ultraviolet-B radiation in *Pisum sativum*: isolation of novel genes by suppression subtractive hybridisation. *Plant Cell Physiol* 2002;43:402–410.
- ▶ 49 Eckl-Dorna J, Klein B, Reichenauer TG, Niederberger V, Valenta R: Exposure of rye (*Secale cereale*) cultivars to elevated ozone levels increases the allergen content in pollen. *J Allergy Clin Immunol* 2010;126:1315–1317.
- ▶ 50 Kim BJ, Kwon JW, Seo JH, et al: Association of ozone exposure with asthma, allergic rhinitis, and allergic sensitization. *Ann Allergy Asthma Immunol* 2011;107:214–219.
- ▶ 51 Inclan R, Gimeno BS, Dizengremel P, Sanchez M: Compensation processes of Aleppo pine (*Pinus halepensis* Mill.) to ozone exposure and drought stress. *Environ Pollut* 2005;137:517–524.