

Exposure to cadmium-contaminated soils increases allergenicity of *Poa annua* L. pollen

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To cite this article: Aina R, Asero R, Ghiani A, Marconi G, Albertini E, Citterio S. Exposure to cadmium-contaminated soils increases allergenicity of *Poa annua* L. pollen. *Allergy* 2010; DOI: 10.1111/j.1398-9995.2010.02364.x.

Keywords

cadmium; grass pollen allergens; *Poa annua* L.; soil pollutants.

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Accepted for publication 9 February 2010

DOI:10.1111/j.1398-9995.2010.02364.x

Edited by: Reto Cramer

Abstract

Background: Pollution is considered as one main cause for the increase of allergic diseases. Air pollutants may cause and worsen airway diseases and are probably able to make pollen allergens more aggressive. Previous studies looked at traffic-related air pollution, but no data about the effects of polluted soils on pollen allergens are available. We aimed to assess the effects of plant exposure to cadmium-contaminated soil on allergenicity of the annual blue grass, *Poa annua* L. pollen.

Methods: *Poa* plants were grown in soil contaminated or not contaminated (control) with cadmium. At flowering, mature pollen was analyzed by microscopy, to calculate the percentage of pollen grains releasing cytoplasmic granules, and by proteomic techniques to analyze allergen proteins. Allergens were identified by sera from grass pollen-allergic patients and by mass spectrometry.

Results: Pollen from Cd-exposed plants released a higher amount of allergenic proteins than control plants. Moreover, Cd-exposed pollen released allergens-containing cytoplasmic grains much more promptly than control pollen. Group 1 and 5 allergens, the major grass pollen allergens, were detected both in control and Cd-exposed extracts. These were the only allergens reacting with patient's sera in control pollen, whereas additional proteins strengthening the signal in the gel region reacting with patient's sera were present in Cd-exposed pollen. These included a pectinesterase, a lipase, a nuclease, and a secretory peroxidase. Moreover, a PR3 class I chitinase-like protein was also immunodetected in exposed plants.

Conclusion: Pollen content of plants grown in Cd-contaminated soils is more easily released in the environment and also shows an increased propensity to bind specific IgE.

Allergic diseases are increasing and it is estimated they will affect half of the population of the European community by 2015, with a full financial impact in Europe of around € 100 billion per annum (<http://www.efanet.org/allergy/documents/EUSummitReportonAllergicDiseases.pdf>). The causes of the increasing incidence of allergies are different and are mainly linked to modern lifestyle. The decreased stimulation of the immune system ('hygiene hypothesis'), an improvement of diagnostic tools, and an increased exposure to new allergens or to indoor/outdoor pollutants are some of the supposed causes (1–3).

Regarding environmental pollution, recent interesting findings suggest that along with a role played in worsening airway diseases (4–6), air pollutants are able to make allergens, especially pollen allergens, more aggressive. It has been shown that chemical pollutants can modify the structure of the pollen

grain, thus facilitating the release of its allergenic content (7, 8), are able to induce post-translational modifications (PTMs) in allergenic proteins (i.e. nitration) (9, 10) and may alter the expression of pollen proteins (11, 12). For instance, Cortegano et al. (11) demonstrated that the expression of Cup a 3, an allergen of *Cupressus arizonica*, increased significantly in pollen of plants grown in polluted areas compared to pollen of plants from areas that were not polluted. In effect, Cup a 3 protein belongs to the PRP (Pathogenesis-Related Proteins) family, whose expression can be regulated in response to environmental stress (including pathogens, chemicals, and temperature). It is interesting to point out that about 25% of plant allergens recorded in the IUIS database, are PRPs (13).

On the whole, these data suggest that pollen of plants exposed to high levels of pollution may induce greater allergic reactions than pollen of plants from rural areas. However,

most studies carried out so far have been mainly focused on traffic-related air pollution and only limited data about the effects of soil pollutants on plant root and leaf allergens are available (14–17). Further no effect of soil contaminants on pollen allergens has been reported yet.

This topic is of great relevance if we consider that plants have a stationary habit and are exposed to soil contaminants during their whole life cycle. For instance, the exposure to soil contaminants might induce the production of organs (i.e. fruits, pollen, leaves, etc) containing modified or differently expressed known allergens or containing new allergenic proteins. Among soil pollutants, heavy metals may be usefully employed to investigate the relationship between soil pollution and allergy, because it is well known that they can modify the plant proteome and are widespread (18–20). Specifically, some heavy metals can regulate the expression of several plant defence proteins (21, 22) some of which are significant cross-reacting allergens (23, 24). For instance, Roth et al. (25) reported that the exposure of plants to cadmium (Cd^{2+}), a very toxic heavy metal, led to the expression of several allergen-like protein by *Arabidopsis thaliana*. Further, PR-3 class I chitinases, which are components of the general plant response against heavy metals (26), were also demonstrated to be allergens because they contain the chitin binding hevein domain, a cysteine-rich 40 amino-acid binding polypeptide, which was firstly identified as a major allergen for patients allergic to latex (27).

In spite of these observations, there are no data about the relationship between heavy metal stress and changes in allergen profile of pollen of common allergenic plants.

In the present study, we investigated the effect of cadmium on pollen allergenic proteins of the annual blue grass, *Poa annua* L. We chose this grass species because it commonly grows in urban areas and because it belongs to the important allergenic plant family *Poaceae*; moreover, molecular information regarding its allergens are missing.

Materials and methods

Plant material and cadmium treatment

Poa annua L. seeds (Tempoverde s.r.l., Turin, Italy) were washed in sterile water and surface sterilized in a 5% chlorine solution (Sigma–Aldrich, St. Louis, MO, USA) for 30 min on a shaking plate. Seeds were then rinsed in sterile water at least three times and were sown in 3% organic matter control soil or in soil contaminated with 50 ppm of Cd^{2+} , added in the form of cadmium sulfate (Sigma St. Louis, MO, USA). Plants were grown in a greenhouse and maintained at 24°C/18°C (day/night) and 60% relative humidity.

After 2 months of treatment, mature pollen grains, naturally released from anthers, were collected.

Pollen samples were analyzed immediately or up to 1 month after collection. During storage, they were kept in sterile tubes at room temperature.

As reference material, certified *Phleum pratense* pollen was purchased by Allergon (Ängelholm, Sweden).

Pollen morphological observation and pollen cytoplasmic granules (PCG) release analysis.

After hydration in distilled water or rainwater, pollen grains release cytoplasmic material (28). The analysis of released pollen cytoplasmic granules (PCG) and of pollen grains collected from control and Cd-exposed *Poa* plants were carried out by means of a Zeiss Axioplan light microscope connected to a Leica DC 300F camera for photograph documentation and with a confocal microscope.

Samples were prepared by placing small amounts of pollen (0.2 mg) on a microscope slide and then re-suspending pollens with a drop (20 µl) of sterile bidistilled water. Slides were observed immediately after sample preparation ($t = 0$) and after 30 min ($t = 30$) of hydration at room temperature. During incubation, the slides were placed on moist filter paper in a closed box to avoid dehydration.

The count of PCG-releasing grains was performed on a total of 1000 grains both for control and treated pollen. At least three independent experiments were carried out.

The acquired digital images were analyzed with the IMAGE-PRO PLUS program (Media Cybernetics, Silver Spring, MD, USA).

Patients

Adult subjects with a history of seasonal, springtime respiratory symptoms who spontaneously presented at the allergy outpatient department of the Clinica San Carlo, Paderno Dugnano, Italy asking for allergy evaluation were considered as potential candidates for the inclusion in this study. All subjects underwent SPT with commercial extracts (Allergopharma, Reinbeck, Germany) of the main seasonal airborne allergens present in Italy, including grass, mugwort, ragweed, pellitory, plantain, birch, olive, and cypress. Readings were taken after 15 min following established methods (29). In order to avoid the interference of cross-reacting pan-allergens such as profilin or polcalcin, sera from nine patients monosensitized to grass pollen were selected for *in-vitro* experiments. All study patients gave an informed consent to venipuncture. Phleum-specific IgE levels in collected sera were measured by ImmunoCAP (Phadia, Uppsala, Sweden) ranging between 9.12 and 89.1 Ku/l. Sera from 3 healthy, nonatopic adults (showing negative clinical history and negative SPT with commercial airborne allergens extracts) were used as controls. All serum samples were aliquoted and stored at –20°C until use.

Preparation of pollen and leaf protein extracts

Soluble protein extracts of *Phleum pratense* L. and *Poa annua* L. pollen were prepared by suspending 0.1 g of pollen in 1 ml of bidistilled sterile water containing different protease inhibitors (Protease Inhibitor Cocktail for general use, Sigma). Samples were incubated on a rotating drum for 1 h at room temperature. The soluble fraction was isolated by means of two centrifugations at 13 000 g for 30 min at 4°C and then stored at –20°C until use.

The extraction of total proteins from *Poa annua* leaves was performed using TRI Reagent® (a mixture of phenol and guanidine thiocyanate in a monophasic solution, Sigma), according to manufacturer's protocol with minor modifications. Briefly, one gram of pooled leaves was frozen in liquid nitrogen, transferred to a prechilled mortar and ground to obtain a fine powder. One milliliter of TRI Reagent® was added to every 100 mg of powdered tissue. After removal of RNA and DNA, proteins were precipitated by isopropanol. The protein pellet was washed three times in 0.3 M guanidine hydrochloride/95% ethanol solution, then centrifuged and washed with 100% ethanol. After centrifugation and removal of ethanol, the pellet was air-dried. The pellet was dissolved in 400 µl of SDS sample buffer [2% (w/v) SDS, 10% (v/v) glycerol, 1 mM DTT, 62.5 mM Tris-HCl, pH 6.8] or in 400 µl of IEF rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 20 mM Tris-HCl, pH 8.8, 20 mM DTT, 0.5% ampholyte mixture carrier, pH 3–10, 0.005% bromophenol blue] for 1D or 2D electrophoresis analysis, respectively. After solubilization, samples were centrifugated at 10 000 g for 10 min at 4°C to remove any insoluble material.

Protein concentration was assayed according to Bradford (30) using bovine serum albumin (BSA) as standard.

SDS-PAGE and immunoblotting

Pollen or leaf extracts (30 µg/lane) were separated by 14% and 12% SDS-polyacrylamide gel, respectively, performed as described by Laemmli (31). Protein extracts dissolved in SDS sample buffer were heated at 95°C for 5 min, loaded on 4% (v/v) acrylamide stacking gel (pH 6.8) and then separated on 14%/12% (v/v) acrylamide running gel (pH 8.8) at 100 V until the dye front reached the bottom of the gel, using a Mini-Protein electrophoresis system (Bio-Rad Laboratories).

Gels were either stained with colloidal Coomassie Blue G-250 (0.1% Coomassie Blue G250, 170 g/l ammonium sulfate, 34% methanol, 3% phosphoric acid) or transferred to nitrocellulose membrane. The molecular weight on gels and blots was determined by using the Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories), covering the range of 10–250 kDa. Moreover, these standards were useful for the transfer efficiency assessment.

For immunodetection of IgE-binding proteins, gels were electroblotted (100 mA, overnight) onto nitrocellulose sheets (0.45 µm; Hybond-C membrane, Amersham) by a Trans-Blot cell (Bio-Rad Laboratories) containing transfer buffer [25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3]. Membranes were blocked with 5% (w/v) nonfat dry milk powder in TBS-T [20 mM Tris, 150 mM NaCl and 0.05% (v/v) Tween 20, pH 7.5] for 1 h and then incubated for 16 h at 4°C with a 1:5 dilution of the sera from grass-allergic patients, and from nonatopic adult individuals as control. Bound IgE were detected using an HRP-conjugated goat anti-human IgE antibody (1:80 000 dilution; Bethyl). Immunoreactive bands were visualized on an X-ray film (Kodak) using SuperSignal West Dura detection kit (Pierce).

For PR3 detection, nitrocellulose membrane were saturated 1 h with blocking solution (5% nonfat dry milk,

TBS-T), rinsed in TBS-T and incubated with 2 µg/ml of anti-tobacco PR3 class I chitinase rabbit polyclonal antibody (Agrisera; <http://www.agrisera.com/en/artiklar/chitinase-pr-3.html>), for 90 min in TBS-T containing 1% (w/v) nonfat milk. After three washes in TBS-T for 10 min each, membranes were soaked for 60 min with sheep anti-rabbit IgG alkaline phosphatase-conjugated (Sigma) diluted in TBS-T [1% (w/v) nonfat milk] at a ratio of 1:15 000. Membranes were then washed three more times with TBS-T. Immunoreactive bands were detected by Sigma Fast BCIP/NBT as alkaline phosphatase substrate according to manufacturer's protocol.

2D electrophoresis and immunoblotting

Proteins extracted from leaf of control or Cd-exposed plants were separated by two-dimensional gel electrophoresis. Isoelectrofocusing (IEF) was carried out on 17-cm long immobilized pH gradient (IPG) strips, providing a nonlinear pH 3–10 gradient (Bio-Rad Laboratories). Strips were rehydrated in 300 µl of rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 20 mM DTT, 0.5% ampholyte mixture carrier, pH 3–10, 0.005% bromophenol blue) containing 400 µg of protein sample. Passive overnight rehydration and IEF were performed at 20°C using a Protean IEF-Cell (Bio-Rad Laboratories). IEF was performed according to the following program of migration: 200 V for 1 h, a slow ramping to 1000 V for 4 h, a linear ramping from 1000 to 10 000 V in 8 h, after which run was continued at 10 000 V to give a total of 76 kVh. The current limit was set at 50 µA/strip.

After the first dimension separation, the IPG strips were equilibrated for 15 min against 6 M urea, 30% glycerol, 2% SDS, 0.375 M Tris-HCl pH 8.8, 2% DTT, in order to resolubilize proteins and reduce disulfur bonds. The -SH groups were then blocked by substituting the DTT with 2.5% iodoacetamide in the equilibration buffer for 15 min. After equilibration, the strips were placed on the top of vertical polyacrylamide gels (14%). Molecular weight markers were run on the acidic hand-side of each gel. An agarose solution (0.5% low melting agarose in running buffer) was loaded onto the gel strips, and electrophoresis was performed at 4°C in a Laemmli running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS) for 30 min at 15 mA/gel, then at 24 mA/gel until the dye front reached the bottom of the gel. Gels, 20 × 20 cm × 1.0 mm, were run in parallel (Protean Dodeca Cell, Bio-Rad Laboratories). For each sample, at least three independent protein extracts and 2DE analyses were performed. Protein detection was performed with colloidal Coomassie Blue G-250 staining.

Alternatively, gels were blotted onto nitrocellulose membrane in a Trans-Blot Cell apparatus (Bio-Rad Laboratories) (100 mA, 16 h, 4°C) and incubated with anti-PR3 antibody, as previously described.

Image processing and analysis system

All gel and blot images were digitalized by a GelDOC 2000 Image Analysis System (Bio-Rad Laboratories). Semi-quantitative

tative analysis of immunoreactive bands or spots was performed by using QUANTITY ONE software 4.5.0 (Bio-Rad Laboratories) to score integrated optical density (IOD) after subtraction of background. At least, three independent measurements on separate blots were done.

In gel digestion and peptide sequencing by Nano-RP-HPLC-ESI-MS/MS

Protein bands were carefully excised from coomassie-stained 1D gels and subjected to in-gel trypsin digestion according to Shevchenko and colleagues (32) with minor modifications.

The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/ μl of trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI, USA) in an ice bath. After 30 min, the supernatant was removed and discarded; 20 μl of 50 mM NH_4HCO_3 were added to the gel pieces, and digestion was allowed to proceed at 37°C overnight. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to mass spectrometric analysis, the peptide mixtures were redissolved in 10 μl of 5% formic acid.

For protein identification by MS/MS, peptide mixtures were separated using a nanoflow-HPLC system (Ultimate; Switchos; Famos; LC Packings, Amsterdam, The Netherlands). A sample volume of 10 μl was loaded by the auto-sampler onto a homemade 2-cm fused silica precolumn [75 μm I.D.; 375 μm O.D.; Reprosil C18-AQ, 3 μm (Ammerbuch-Entringen, DE, USA)] at a flow rate of 2 $\mu\text{l}/\text{min}$. Sequential elution of peptides was accomplished using a flow rate of 200 nL/min and a linear gradient from Solution A (2% acetonitrile; 0.1% formic acid) to 50% of Solution B (98% acetonitrile; 0.1% formic acid) in 40 min over the precolumn in-line with a homemade 10–15-cm resolving column (75 μm I.D.; 375 μm O.D.; Reprosil C18-AQ, 3 μm (Ammerbuch-Entringen, Germany)).

Peptides were eluted directly into a High Capacity ion Trap (model HCTplus, Bruker-Daltonik, Germany). Capillary voltage was 1.5–2 kV, and a dry gas flow rate of 10 l/min was used with a temperature of 230°C. The scan range used was from 300 to 1800 m/z.

Proteins were identified by searching the National Center for Biotechnology Information nonredundant database (NCBI nr, version 20090606, <http://www.ncbi.nlm.nih.gov>) using the Mascot program (in-house version 2.2, Matrix Science, London, UK). The following parameters were adopted for database searches: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide Mass Tolerance $\pm$ 1.2 Da, Fragment Mass Tolerance $\pm$ 0.9 Da, missed cleavages 2. For positive identification, the score of the result of $[-10 \times \text{Log}(P)]$ had to be over the significance threshold level ($P < 0.05$).

Statistics

Data were statistically analyzed by STATGRAPHICS PLUS program for Windows (Manugistic, MD, USA): Student's *t*-test, for two-sample comparison, or ANOVA and Duncan test, for

multiple sample comparison, were applied when normality and homogeneity of variance were satisfied. Data, which did not conform to the assumptions, were alternatively transformed into logarithms or were analyzed by Mann–Whitney or Kruskal–Wallis nonparametric procedures (for two or multiple sample comparison, respectively).

Results

Light microscope analysis of PCG release

In our experiments, pollen samples collected and stored for 24 h were analyzed for their ability to release PCG by traditional and confocal light microscopy. PCG release was measured just after the contact of pollen with water ($t = 0$) and after 30 min of hydration ($t = 30$). Figure 1A shows an example of a PCG-releasing *Poa annua* pollen grain. At $t = 0$, the mean percentage of pollen grains releasing PCG was higher in pollen samples from plants exposed to 50 ppm of Cd^{2+} (3.3%) than in samples from control plants (2.0%, Fig. 1B), although the difference did not reach statistical significance ($P = 0.064$). The difference became significant ($P < 0.05$) after 30 min of hydration, when the mean percentage of PCG releasing pollen from exposed plants reached 13.4%, whereas that from control plants increased only slightly to 4.3% (Fig. 1B).

IgE reactivity to Phleum pratense pollen extract by patients' sera

The grass pollen IgE reactivity of sera from the 9 patients with grass-pollen allergy was tested by immunoblot to *Phleum pratense* extract (Fig. 2). *Phleum* proteins were separated by SDS-PAGE and blotted on nitrocellulose membrane, and each lane was probed with one of the 9 allergic patients' sera, diluted 1:5. All sera (100%) showed IgE binding to a protein band with apparent molecular masses of 30–32 kDa, most likely the group 1 grass major allergen (Phl p1, β -expansin). Allergens with molecular masses under 20 kDa were detected by 44.5% (4 of 9) of these sera. The recognition rate for allergens with apparent molecular masses of 38, 50, and 60 kDa was low (22–33%). No IgE binding was seen when the serum from control subjects were used (neg.).

Allergen detection in pollen extracts from control and treated Poa plants

Soluble pollen proteins extracted from control and Cd^{2+} -exposed *Poa annua* plants were separated by SDS-PAGE. Figure 3A shows an example of coomassie-stained gel: differences between protein profiles of control and treated samples can be observed.

For the identification of allergenic molecules, proteins were transferred onto nitrocellulose filter and incubated with a pool of the selected patients' sera. Immunoblots revealed a 30-kDa band recognized by sera in both control and Cd-exposed samples (Fig. 3B). However, in Cd-exposed pollen extracts, the signal at 30 kDa was much stronger and other additional overlapping signals ranging from 30 to 34 kDa

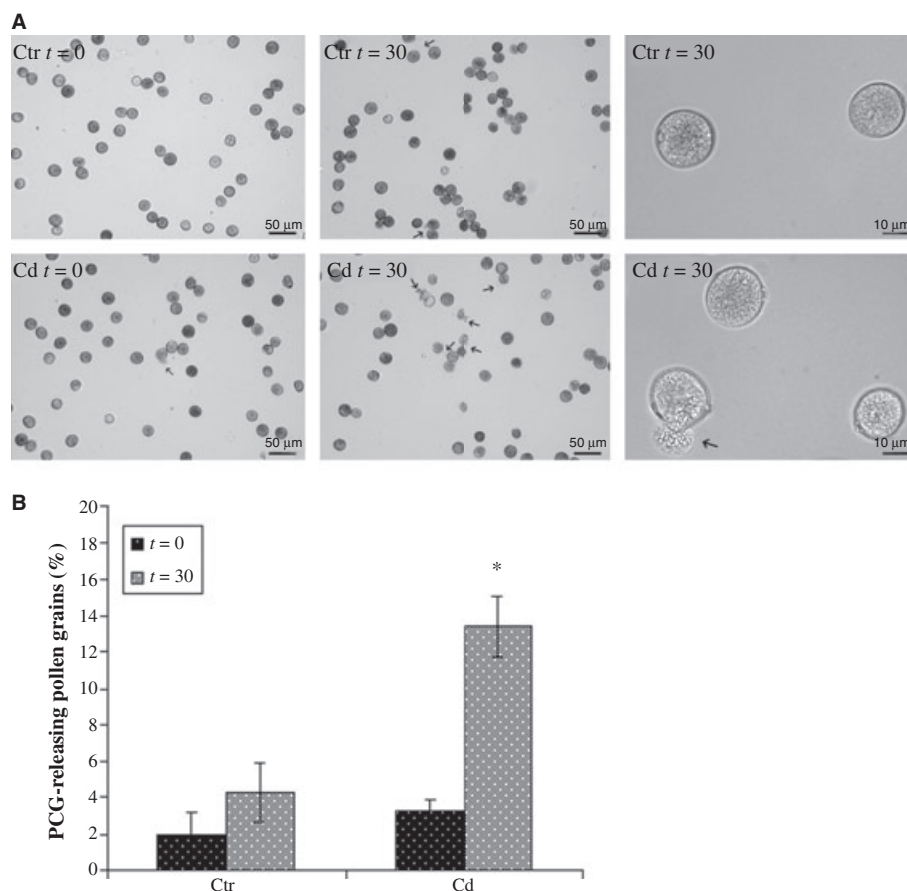


Figure 1 (A) control (Ctr) and Cd-exposed (Cd) *Poa annua* pollen grains immediately after water contact ($t=0$) and after 30' of hydration ($t=30$) in sterile bidistilled water. Pollen cytoplasmic granules (PCGs) are indicated by arrows. (B) Percentage of PCG-

releasing pollen grains in control and exposed samples, measured at $t=0$ and $t=30$. The asterisk (*) indicates a statistically significant difference with the control ($P < 0.05$).

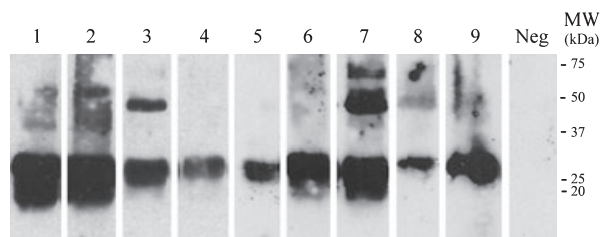


Figure 2 Representative example of IgE reactivity by immunoblot using sera from grass-sensitized patients and *Phleum pratense* pollen extracts. Neg: control serum from nonatopic adult individuals.

were detected. No other reactive proteins were detected on the filters. It is noteworthy that presently no protein sequences are recorded for *Poa annua* in allergen databases such as IUIS, or Allergome.

Sequence analysis

In order to identify the proteins recognized by the patients' sera, the regions of the preparative 1D gels, corresponding to

the signals detected in immunoblots of Cd-exposed and control sample, were excised and analyzed by mass spectrometry (Nano-RP-HPLC-ESI-MS/MS).

Protein identification was carried out by ProFound and MASCOT using DB searches including sequences from Eukaryota organisms or sequences from Viridiplantae (green plants).

Table 1 reports the list of the identified proteins. In control (Ctr), only two proteins, belonging to the grass allergen groups 1 and 5, were found. The same allergens were revealed in Cd-exposed pollen samples. These proteins are the first allergens identified in *Poa annua* and are expressed irrespectively in the presence or absence of cadmium. By contrast, five additional proteins were specifically identified at 30–35 kDa by patients' sera in Cd-exposed pollen extract (Fig. 3B, Table 1). Most of these proteins are related to the plant response to biotic and abiotic stresses and their homologues in other species have been proved to be allergens.

In parallel to mass spectrometry, an antibody against PR3 class I chitinase was used to test the presence of PR3-like proteins in the pollen extracts because these proteins are allergens with a molecular mass ranging from 25 to 35 kDa

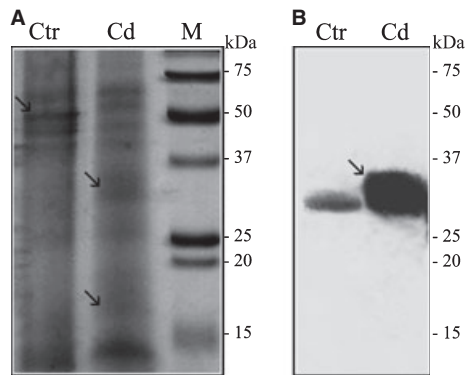


Figure 3 Allergen detection in *Poa annua* pollen extracts from control and cadmium-exposed plants. (A) Coomassie blue stained 1D gel of soluble pollen protein extracts and (B) related immunoblots probed with a pool of selected patients' sera (1:5 sera dilution; HRP detection). Differences in protein profiles and antibody labeling are indicated by arrows.

and their expression is induced by heavy metals (26, 33). Protein extracted from control, Cd-exposed pollen and leaf samples were separated by 1D SDS-PAGE. Leaf extracts were also analyzed by 2DE. Proteins were blotted onto nitrocellulose membrane and probed with the anti-PR3 class I chitinase antibody. Figure 4 shows a representative example of 1D Western blots. The presence of PR3-like proteins was revealed in pollen extracts of plants exposed to cadmium but not in control (Fig. 4A). The proteins were detected in the gel region recognized by patients' sera. The higher expression of PR3 in Cd-exposed plants was confirmed by the analysis of leaves: a single band at 32 kDa was observed in leaf extracts both from control and exposed plants (Fig. 4B), but the signal was statistically greater ($P < 0.05$) in exposed sample.

To further investigate the presence of different PR3 isoforms in control and exposed leaves, two-dimensional immunoblotting was performed (Fig. 5). Two main spots at 32 kDa, showing different isoelectric points (pI), were detected. In treated samples, the signals were higher and a

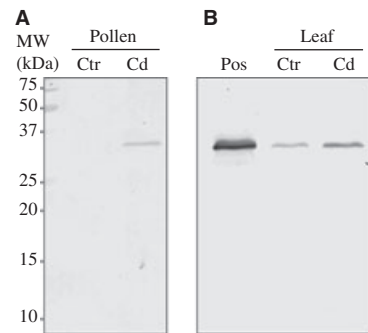


Figure 4 Detection of PR3-like proteins by anti-tobacco class I Chitinase antibody. A: pollen extracts from control and exposed *Poa* plants. B: leaf extracts from control and exposed plants. Pos: positive control (*Prunus persica* chitinase-like protein purified from fruit treated with ethylene).

third, more acidic isoform was revealed. Statistical analysis of the mean integrated optical density (IOD) of the immuno-reactive spots revealed significant differences between control and treated samples (Fig. 5C).

Discussion

In recent years, the study of the effects of pollution on pollen allergenicity has arisen great interest. Published data suggest that pollutants can induce structural modifications of the pollen grain (8), may influence the expression of pollen proteins (11) (i.e., PR-proteins induction), or induce post-translational modifications (PTMs) of allergens (9). These observations suggest that exposure to pollutants may eventually result in an increase of allergenic potential of pollen. Nevertheless, up to now, studies have been mainly focused on air pollutants (34–36), whereas the effect of soil contaminants has been investigated only in plant vegetative organs (14–17). These last studies, in particular, demonstrate that soil heavy metals affect plant proteome and increase the content of defence protein such as PR-10. However, regarding the effect of soil contaminants on pollen allergens,

Table 1 List of proteins identified by MS in the pollen extract gel regions reacting with allergic patient sera. Cd: cadmium-exposed plants; Ctr: control plants

	Mw kDa theor.	pI pred.	No. of peptides identified	Mascot Score	NCBI Accession Number	Protein ID
Ctr	37798	9.45	5	332	gil113560	Pollen allergen KBG 31 (Pollen allergen <i>Poa</i> p9, re-named <i>Poa</i> p5)
	28728	6.46	4	243	gil3860384	Major group I allergen Hol I 1 [<i>Holcus lanatus</i>] (95% identity with <i>Poa</i> p1)
Cd	28627	6.45	3	211	gil4090265	Group I pollen allergen [<i>Poa pratensis</i>], <i>Poa</i> p1
	37798	9.45	4	183	gil113560	Pollen allergen KBG 31 (Pollen allergen <i>Poa</i> p9, re-named <i>Poa</i> p5)
	33976	9.19	2	99	gil75319702	Pectinesterase [<i>Nicotiana plumbaginifolia</i>]
	36956	8.22	3	122	gil55296706	Putative lipase [<i>Oryza sativa</i> Japonica Group]
	38283	6.23	2	100	gil162458978	Legumin-like protein [<i>Zea mays</i>]
	32686	7.57	2	92	gil13274190	Putative nuclease [<i>Hordeum vulgare</i> subsp. vulgare]
	39264	5.78	2	73	gil226495501	Peroxidase 65 [<i>Zea mays</i>]

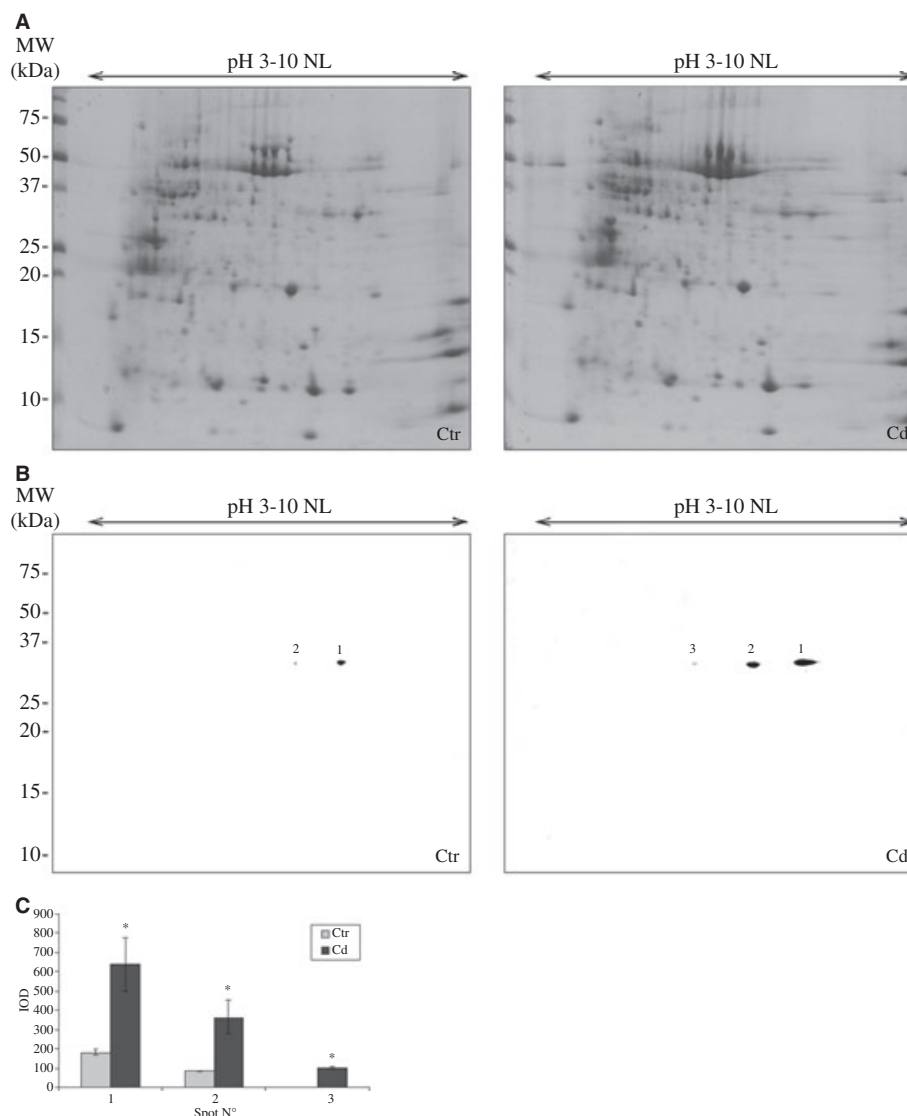


Figure 5 Example of coomassie-stained 2D gels (A) and the related immunoblots (B) with anti-PR3 antibody of leaf extracts from control (Ctr) and exposed (Cd) plants. (C) Quantitative analysis of immunoreactive spots. Results are the mean of three different

experiments $\pm$ standard error (SE). (*) Statistically significant difference from the relative control ($P < 0.05$). IOD = Integrated Optical Density.

no data is available. In the present study, we evaluated for the first time the effect of cadmium-contaminated soils on allergenic pollen. We chose the annual bluegrass (*Poa annua* L.) as a model plant for this study because it belongs to the *Poaceae* plant family, the most frequent cause of seasonal respiratory allergy worldwide (37). *Poa annua* is considered a relevant allergen source (38), although no allergens have been isolated, characterized and recorded into official allergens databases so far. *Poa annua* is a widespread low-growing annual plant in temperate climates and is one of the most common grasses in urban areas, growing in flowerbeds, close to the tram tracks, and along roadsides where it is directly or indirectly easily exposed to pollutants (39).

We exposed *P. annua* plants to Cd by sowing seeds on Cd-contaminated soil. At flowering, we collected pollen from Cd-exposed plants as well as from control plants.

We selected cadmium as a model compound for the study of the effects of heavy metal pollution of the soil on pollen allergenicity, because it is an important pollutant largely present in urban soils because of the fallout of air particulate matter (vehicular emissions, incinerators, etc.) and to the use of fertilizers in public parks (40). The natural level of cadmium in soils is usually less than 1 ppm, but it can reach hundreds of ppm in contaminated soils. Thus, the cadmium concentration we used in our experiments is consistent with the values found in polluted areas, where *Poaceae* plants easily grow (41).

In this study, we were able to demonstrate that pollen produced by plants grown in Cd-contaminated soils releases a significantly higher amount of allergen proteins reacting with IgE from *Poa* allergic patients compared to control plants. We also demonstrated that Cd-exposed pollen is more prone to release cytoplasmic grains, which usually contain the allergenic particles. This last finding is in keeping with previous observations made for vehicular pollutants, which increase the PCG release (8).

The identification of the proteins present in the gel region reacting with patients' IgE by mass spectrometry revealed that both control and Cd-exposed plants contain and release the two major grass pollen allergens belonging to groups 1 and 5. These are the first allergens identified in *Poa annua* and represent the only proteins reacting with patients' sera detected in control pollen. As group 1 and 5 grass allergens are usually associated with PCGs (42), and the main mechanisms involved in pollen allergen release are protein diffusion through the cell wall (43) and PCG release through the germination pore (44), it can be supposed that the higher proportion of PCGs-releasing pollen grains found in exposed plants contributes to the stronger allergen reactivity. However, proteins other than group 1 and 5 allergens, which might have contributed to strengthen the signal, were identified in Cd-exposed pollen. These include a pectinesterase, a lipase, a nuclease and a secretory peroxidase-like protein (Table 1). These proteins are not included among grass allergens reported in the main databanks, although their role in plant defence mechanisms suggests their potential allergenicity (13, 23, 45, 46). For instance, pectinesterase has a role in plant stress defence (47) and shows a high homology (51% sequence identity) with vegetable food allergens such as Act d 7, a 35 kDa kiwi protein (Uniprot P85076), and Lyc PME in tomato. Further, by the use of a technique that is more sensitive than mass spectrometry, in Cd-exposed pollen we detected an additional allergen candidate, a 32-kDa PR3 class I chitinase-like protein. PR3 class I chitinases are plant proteins whose expression can be increased by biotic or abiotic stress and represent a family of allergens (26, 48, 49).

The analysis of Cd-exposed and control leaves confirmed that Cd-polluted soil increases the expression of this PR3-like protein and showed that two PR3-like isoforms were shared by exposed and control leaves, whereas a third, more acidic, isoform was specifically found in exposed leaves, and likely corresponded to that detected in Cd-exposed pollen.

In conclusion, our data show that soil pollutants, such as cadmium, are able to increase the allergenicity of pollen, although the mechanisms leading to this are not yet clear. For sure, pollutant-exposed pollen is more prone to release PCG and, hence, it may release a greater amount of allergens. Further, the expression of new allergens related to the synthesis of plant stress proteins should be considered, as in our experiments some defence proteins homologous to known allergens were specifically induced by cadmium and were detected in the gel region reacting with patients' sera. Our findings have clear clinical implications as they might provide a reasonable explanation for the impressive increase of allergic diseases that has occurred in the developed areas of the world during the last decades, that is the release of higher amounts of pollen, that is also intrinsically more allergenic, by plants grown in polluted areas.

Future studies will define which proteins and which isoforms are responsible for the increased allergenicity of pollen, and will clarify the mechanisms involved.

Acknowledgments

This work was supported by the Italian Ministry of University and Research and by a grant from Fondazione Banca del Monte di Lombardia. The Authors thank Professor Lello Zolla and Dr Maria Giulia Egidì for mass spectrometry analyses.

Sources of funding

This work was funded by the Italian Ministry of University and Research and by a grant from Fondazione Banca del Monte di Lombardia.

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