



# Traffic-related NO<sub>2</sub> affects expression of *Cupressus sempervirens* L. pollen allergens

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## Abstract

**Introduction and Objective.** Traffic pollution has been recognized as directly worsening respiratory symptoms of allergic subjects, although whether urban air pollutants can also directly increase the allergenic potential of pollen has not yet been definitely proven. Therefore, the hypothesis that intra-urban air NO<sub>2</sub> variation influences allergens expression in *Cupressus sempervirens* (Cs) L. pollen was tested.

**Material and methods.** Mature microsporophylls were cut from Cs trees of similar age and height (14–17 m) present in three different sites of Florence (Italy) and processed in the laboratory. Cs pollen allergens amount was determined by a semi-quantitative analysis of electrophoretically separated pollen extracts fractions. NO<sub>2</sub> air concentrations were recorded by air monitoring stations located at a distance not exceeding 50 m from each pollen collection site, and the relative annual mean values were acquired by a publicly available database (Tuscan Regional Agency for Environment Protection).

**Results.** Expression of three major Cs pollen allergens was non-linearly correlated with mean annual NO<sub>2</sub> concentrations. Expression peak of all major allergens considered was reached at NO<sub>2</sub> air concentration (67µg/m<sup>3</sup>), far below the value at risk for direct effect on the respiratory health (European Union Directive 2008/50/EC).

**Conclusions.** The findings suggest that intra-urban NO<sub>2</sub> variations do affect the expression of Cs pollen major allergens, and an apparent low risk NO<sub>2</sub> concentration should be regarded as indirectly harmful for increasing the allergenic potential of pollen.

## Key words

urban air pollutants, NO<sub>2</sub>, Pollinosis, pollen allergens, *Cupressus sempervirens* L.

## INTRODUCTION

Allergic rhinitis and asthma have a significant economic impact on patients, patients' families and society as a whole [1]. The growing prevalence of allergy also has major economic consequences for society due to absence from education and work or impaired performance, thereby placing a greater burden on healthcare resources and increasing medication costs [1]. For example, mean annual direct and indirect costs due to respiratory allergic diseases in Italy in 2013 were estimated to be 7.34 billion Euros [1]. Although recognized as the result of an interaction between multiple genetic and environmental factors [2], allergies are mainly considered as an environmental disease [3]. Indeed, the dramatic increase in allergy-related diseases observed in the past decades cannot be ascribed only to genetic factors [3]. Allergy to Cs pollen is widely diffused in the Mediterranean area [4, 5]. Since major Cs allergens are highly cross-reactive with allergens of both species of the same genus and species belonging to other genera (i.e. *Hesperocyparis*, *Platyclusus*, *Cryptomeria*, *Chamaecyparis*, *Juniperus*), the prevalence of this allergy can depend on the geographic area and diffusion of allergenic

genera [5]. In fact, the prevalence of Cs allergy in Italy is 18% of all allergic population, with a 28% prevalence peak in the regions of Tuscany and Umbria [6], and 43% in Lazio region [7] where the presence of Cs is significant in both rural and urban areas [8, 9], and it is increasing in metropolitan areas [10].

Several *in vitro* studies have shown that the bioavailability of allergens is directly influenced by air pollution [11]. In particular, recent data [12–16] indicate that exposure of allergenic plants to NO<sub>2</sub> gas can increase the expression of pollen allergens and/or the allergenicity of the pollens. Atmospheric NO<sub>2</sub> has long been known to be harmful both to plants [17] and humans [18]. To our knowledge, no data on NO<sub>2</sub> effect on Cs pollen allergenic potential are available so far.

## OBJECTIVES

We hypothesize that *Cupressus sempervirens* L.(Cs) plants exposure to traffic-related air pollution, particularly to the NO<sub>2</sub> gas component, could be associated with an increased allergenic potential of its pollen. Starting from these premises, this study focusses on assessing whether the Cs pollen major allergens were differently expressed in association to traffic-related NO<sub>2</sub> air pollutant, comparing pollen samples from differently polluted sites in the city of Florence (Italy).

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## MATERIALS AND METHOD

**Pollen collection and quantification.** Pollen of *Cs* was collected during the flowering season of 2011 in the city of Florence, Italy, where *Cupressus sempervirens* L. released pollen from 1 February – 5 April. The peak occurred on 7 March when 8,944 pollen granules/m<sup>3</sup> of air were sampled with a pollen catcher (volumetric sampler type Hirst, model VPPS 2000, Lanzoni, Bologna, Italy). The day of sampling, 16 February 2011, corresponded to the beginning of the pollen grains exponential release phase (1,958 granules/m<sup>3</sup>), when most of the sporophylls were ripe but still sufficiently rich in pollen grains (<http://sira.arpat.toscana.it/sira/>). To exclude the influence of temporal and climatic variables, all pollen samples were collected on the same sunny day (16 February) at all the city sites. Three city sites characterized by medium-high traffic intensity were selected, in each of which were selected three *Cs* trees (less than 5 m apart from each other) of similar age (30–40 years) and height (14–17 m), planted by municipal services.

Air monitoring stations are located at a distance not exceeding 50 m from each pollen collection site (Fig. 1). The site names, corresponding to the nearest street name, were Bassi, Ponte alle Mosse (Mosse hereafter) and Gramsci. Mapped urban forests characterized by the presence of conifers are present in the indicated sites [19].

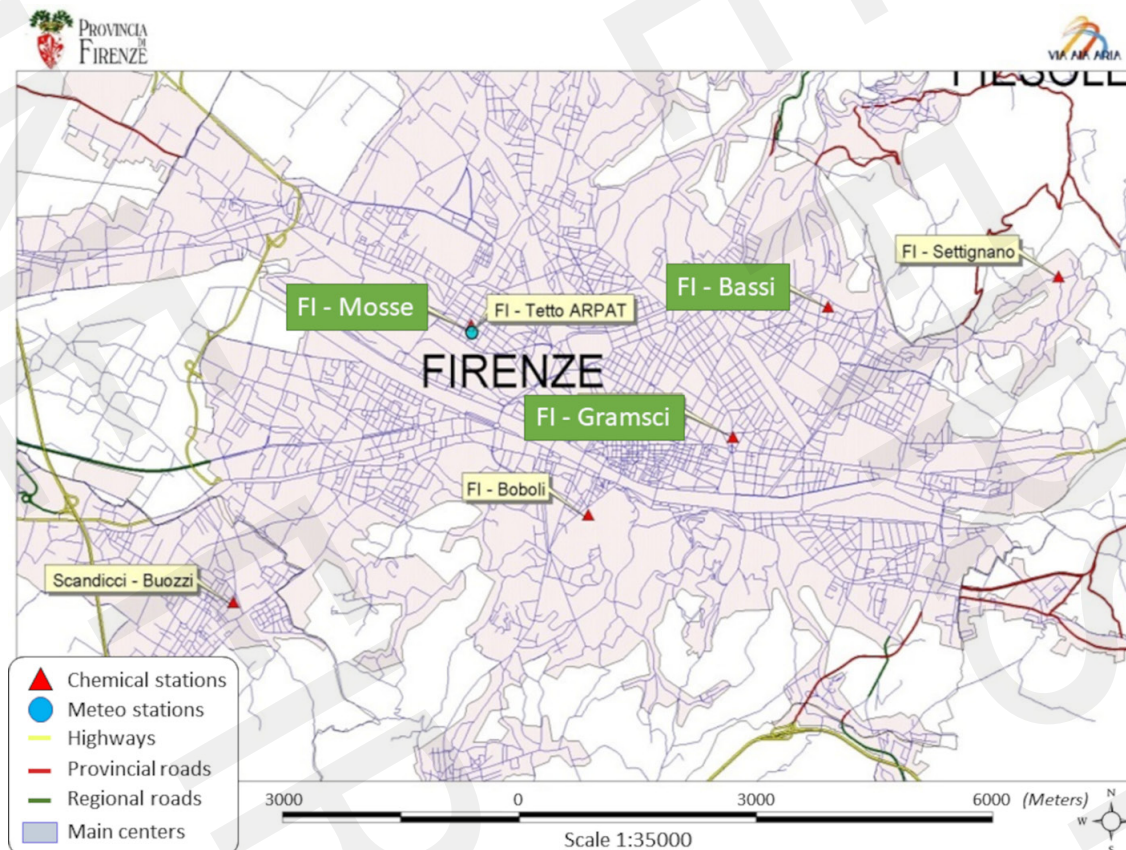
Eight twigs per *Cs* tree, bearing mature microsporophylls from the medium and the lower part of the crown were randomly cut from different orientations, placed in plastic bags and processed in the laboratory within two hours.

The *Cs* twigs were arranged in vases placed on a sheet of wrapping paper and maintained at 25 °C to collect the shed pollen, as described in Barberini et al. [4]. The collected pollen was sieved with a 300 µm sieve to separate extraneous materials and then dehydrated in a laboratory dryer at room temperature (18–20 °C) using silica gel, until 30–35% of relative humidity (RH) was reached. The pollen was then stored at -20 °C in hermetically-sealed plastic tubes until the analyzes.

In two of the three trees per site, the total amount of microsporophylls and the total pollen produced per square meter of crown was estimated, as in [20] and [21]. Briefly, a flexible square (1 m<sup>2</sup>) was randomly leaned to the lower part of each tree crown (three replicated squares per tree), and all the twigs bearing microsporophylls inside the square were collected and processed as previously described. The resulting pollen was weighted by a Mettler College 150 Digital Precision Laboratory Balance.

**Pollen proteins extraction.** Pollen grains of *Cs* from each tree and from each city site were separately incubated overnight at room temperature (r.t.) in a rotating stirrer in phosphate buffer saline (PBS) pH 7.4 (10%, w:v) with 1% (v:v) of protease inhibition mixture (Halt protease Inhibitor Cocktail 87785; Thermo Fisher Scientific, Waltham (MA), USA) and 0.001% (v:v) of ethylenediaminetetraacetic acid (EDTA).

After centrifugation (18,000 g, 4 °C, 20 minutes), the supernatant was collected and stored in aliquots at -20 °C until use. *Cs* pollen grains were purchased (Allergon AB; Valingeveggen 309, SE-262 92 Angelholm, Sweden) and



**Figure 1.** Distribution of air monitoring stations in the city of Florence. The planimetric map displays the location of the air quality monitoring stations inside the area of Florence. Symbols and colors depict the type of monitoring stations indicated in the planimetric map. White text in green background rectangles denotes the investigated air monitoring stations.



extracted according to the same procedure and used as control sample. Protein concentration in the supernatants was measured by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

**One-dimensional gel electrophoresis and immunoblotting.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% (w:v) polyacrylamide under reducing conditions with a Mini-Protean apparatus (Bio-Rad Laboratories; 1000 Alfred Nobel Drive, Hercules (CA), USA). The gel was then stained with 0.05% Coomassie Brilliant Blue (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in water/methanol/acetic acid (50:40:10). SDS-PAGE and immunoblotting experiments were carried out, as previously described [22, 23]. After electrophoresis (10 µg protein/well), proteins extracted from *Cs* pollen grains collected in the different city sites (Bassi, Mosse, Gramsci) were transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany).

Membranes were blocked with 3% (w:v) gelatin in PBS, then incubated overnight at room temperature (r.t) with serum from a *Cs* allergic patient [PlasmaLab International, Everett (WA), USA] diluted in 0.05% Tween 20 in PBS (PBS-T). Human IgE were detected by peroxidase-conjugated goat anti-human IgE antibody [KPL, Gaithersburg (MD), USA]. The 3,9-diaminobenzidine peroxidase substrate (Sigma-Aldrich, Milan, Italy) was then added to develop the colourimetric reaction.

**Gel and nitrocellulose images digitization and semi-quantitative determination of allergens expression.** SDS-PAGE gel and nitrocellulose sheets containing *Cs* pollen proteins were scanned for documentation and analysis by an UVIDoc scanner (Cambridge, UK). Photographs were acquired in TIFF format for further analysis by using the ImageJ software (<https://imagej.net/Welcome>), developed by the National Institutes of Health [Bethesda (MD), USA].

The original SDS-PAGE images were then used to compute the associated density profile images. A standard curve was constructed by plotting the log molecular weights of each protein marker (Prestained Protein SHARPMASS™ VI, Cod. EPS025500 Euroclone SpA, Italy) on y-axis against their relative forward (Rf) values on x-axis. Subsequently, the standard curve regression was used to compute the estimated molecular weight (MW) of the unknown *Cs* bands on each density profile.

The amounts of the electrophoretically separated fractions (Cup s-39 kDa, Cup s-47.8 kDa and Cup s-97.8 kDa) were determined by densitometric analysis of SDS-PAGE band intensities and width using the ImageJ software. These amounts were expressed as integrated density area (IDarea) values. Protein expression levels determined for each *Cs* allergen were normalized to bovine serum albumin (BSA) and reported as arbitrary units (A.U.), calculated as:

$$A. U. = (ID \text{ area of allergen band} / ID \text{ area of } 250 \text{ ng loaded BSA band}) \times 10^3$$

**Air quality data.** Data of mean annual NO<sub>2</sub> concentrations were collected for the sampling year 2011 from the Tuscan Regional Agency for Environment Protection (ARPAT) database (<http://sira.arpat.toscana.it/sira/>) from the nearest

air monitoring stations. NO<sub>2</sub> monitoring stations placed in Gramsci and Mosse are classified as 'Traffic', the monitoring station located in Bassi is classified as 'Background'. 'Traffic' means that the station is located in such a position that the level of pollution is mainly influenced by traffic emissions from neighbouring roads with medium-high traffic intensity. 'Background' means that the station is located in a site where the level of pollution is not mainly influenced by emissions from specific sources (industry, traffic, residential heating, etc.). The coordinates (Gauss Boaga projection) of NO<sub>2</sub> monitoring stations are: Mosse – N:4850406 – E:1679502, Gramsci – N:4849080 – E:1682817, Bassi – N:4850623 – E:1684020.

**Statistical and regression analysis.** To compare mean values of atmospheric NO<sub>2</sub> concentration and pollen allergens expression at the city sites, One-way ANOVA and *post-hoc* Tukey test for multiple comparisons were performed. Previous reports [15], [16] showed a functional correlation between pollen grains protein expression and NO<sub>2</sub> air concentration in plants exposed to this gas throughout the entire growing season. Based on this, for Cupressus plants exposed to NO<sub>2</sub> for a long time, mean annual NO<sub>2</sub> concentration value was considered as the nearest to real exposure concentration. A non-linear regression analysis was conducted to determine the better fitting curve.

## RESULTS

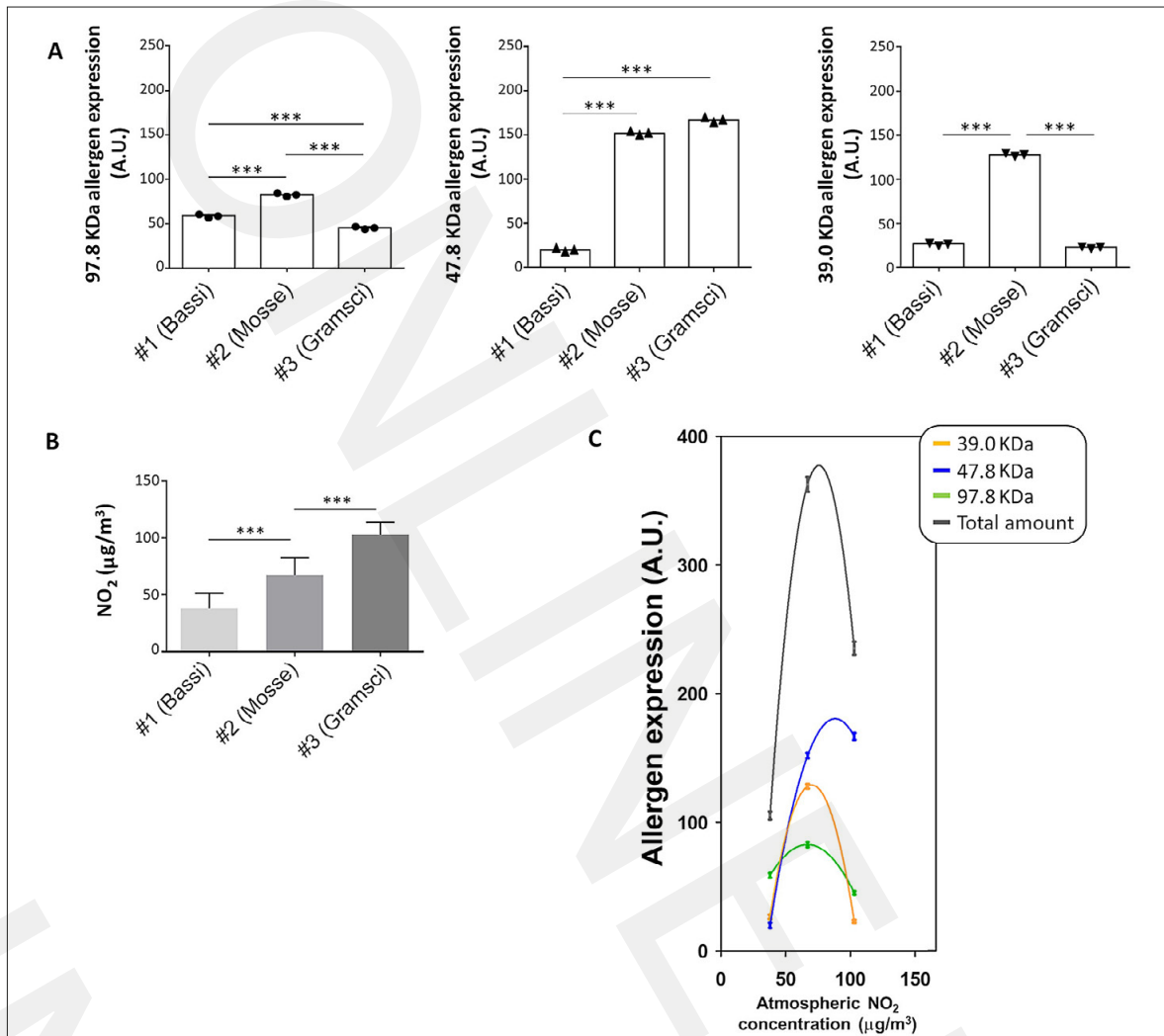
NO<sub>2</sub> mean annual concentration values at the three sites were significantly different ( $p < 0.0001$ ) in the range of 38–103 µg/m<sup>3</sup> (at #1-Bassi and #3-Gramsci, respectively). Moreover, data indicate that site #3 (Gramsci) was more polluted than site #1(Bassi), with site #2 (Mosse) showing intermediate values (Tab. 1, Fig. 2B). The PM10 values are indicative of vehicular traffic being the main NO<sub>2</sub> source at these sites. Analysis of the SDS-PAGE results reveals a polypeptide profile of proteins extracted from *Cs* pollen grains composed of several bands with molecular weights ranging between 100 kDa and 17 kDa (Fig. 3A). Serum IgE recognized the most relevant allergenic components of 39 and 47.8 kDa, corresponding respectively to the Cup s 1 and Cup s 2 allergens [24] and the 97.8 kDa component in the *Cs* pollen extract (Fig. 3B).

**Table 1.** Air pollutants and pollen sampling sites

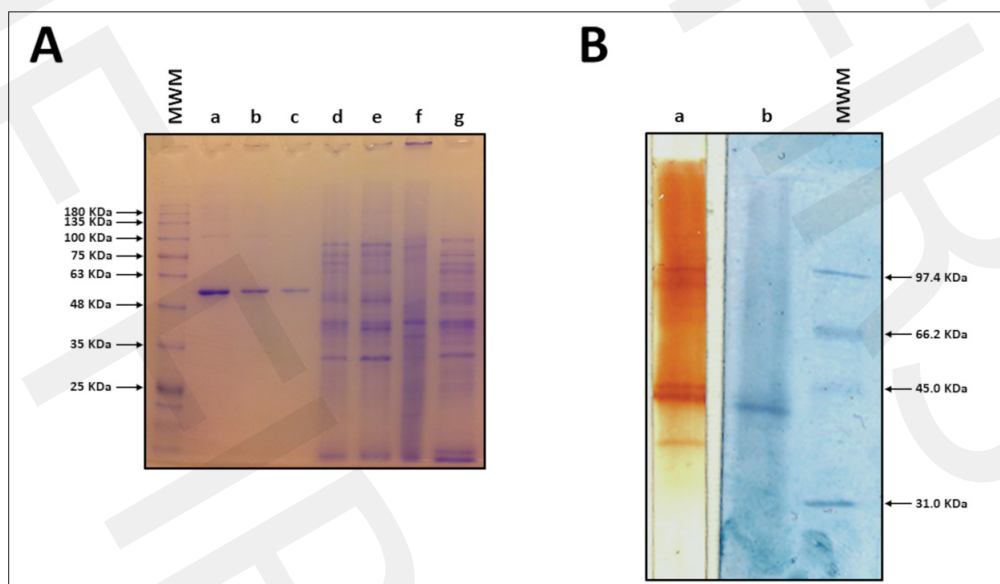
Pollen code	City site	Site classification	PM10 (µg/m <sup>3</sup> )	NO <sub>2</sub> (µg/m <sup>3</sup> )
#1	Bassi	Urban Background	23	38
#2	Mosse	Urban Traffic	38	67
#3	Gramsci	Urban Traffic	38	103

Site name, classification of air monitoring stations (according to <http://data.europa.eu/eli/dir/2008/50/oj>) and mean annual air concentration of NO<sub>2</sub> and PM10 in the sampling year 2011.

The amount of all three major *Cs* allergens changed significantly in relation to the site from which they were collected. In particular, the highest values ( $p < 0.0001$ ) were observed at the #2-Mosse site, both for the 97.8 kDa ( $82.77 \pm 1.904$  A.U.) and the 39 kDa ( $128.14 \pm 1.780$  A.U.) allergens. On the contrary, the 47.8 kDa allergen was highly



**Figure 2.** Model for NO<sub>2</sub>-allergens expression relationship. **A.** Changes of Cs allergens expression (mean ± S.D. of arbitrary units determined by three SDS-PAGE replicates). \*\*\* P<0.001. **B.** Changes of NO<sub>2</sub> air concentration (mean ± S.D. of µg/m<sup>3</sup>). **C.** Graphs describe allergen amount changes in relation to NO<sub>2</sub> air concentration



**Figure 3.** Representative Cs pollen extracts SDS-PAGE and immunoblot. **A.** Lowercase letters represent lanes of the SDS-PAGE. Lanes a-c: BSA 500, 250 and 125 ng, respectively; Lane d, Cs#1; Lane e, Cs#2; Lane f, extract from commercial Cs pollen; Lane g, Cs#3. **B.** Immunoblot of Cs#2 sample with serum IgE derived from an allergic subject. MWM, molecular weight markers. Arrows in each panel indicate the values associated to each MWM band

**Table 2.** Pollen allergen amount changes in relation to NO<sub>2</sub> air concentration, measured at the different pollen sampling sites

Pollen sample	97.8 kDa MW allergen (A.U.)	47.8 kDa MW allergen (A.U.)	39 kDa MW allergen (A.U.)	Total amount of major allergens (A.U.)	Atmospheric NO <sub>2</sub> concentration <sup>§</sup> (µg/m <sup>3</sup> )
#1	58.83 ± 1.850	19.98 ± 1.990	26.67 ± 1.528	105.48 ± 3.051	38.22 ± 13.20
#2	82.77 ± 1.904	151.93 ± 2.046	128.14 ± 1.780	362.84 ± 5.728	67.50 ± 15.08
#3	45.36 ± 1.350	166.97 ± 2.800	23.13 ± 1.172	235.46 ± 5.114	103.2 ± 10.34

97.8 kDa, 47.8 kDa and 39 kDa allergenic fractions amount ± S.D., calculated as in Materials and Method. Three replicated SDS-PAGE of pollen extracts were considered for calculating the indicated values. A.U., arbitrary units; <sup>§</sup>2011 annual mean values.

expressed at the #3-Gramsci site (166.97 ± 2.8 A.U.;  $p < 0.0001$ ) (Tab. 2, Fig. 2A). Considering the total amount of the three major allergens, the highest allergenic expression (362.84 A.U. ± 5.728) was observed at # 2-Mosse (Tab. 2, Fig. 2C). Among the different sites, the amount of each single allergen expressed in pollens displayed a variation between 2 and 8-fold (Tab. 2). Furthermore, it was observed that protein expression level of all three major Cs pollen allergens increased non-linearly with increasing NO<sub>2</sub> concentration. In particular, the obtained curves show an inverse 2<sup>nd</sup> order polynomial trend for all three allergens (Fig. 2C, Tab. 3). It was not possible to evaluate the effect of PM<sub>2.5</sub> and O<sub>3</sub> as fundamental air concentration data were not entirely available for the three city sites. The mean amount of pollen grains produced per square meter of cypress crown was significantly different in the three sites ( $p = 0.0075$ ): lower in Bassi (14.7 g/m<sup>2</sup>), higher in Gramsci (27.45 g/m<sup>2</sup>, while in Mosse showed intermediate values (18.3 g/m<sup>2</sup>).

**Table 3.** Regression analysis data. Statistical parameters and coefficients from the nonlinear regression computed for each allergen are reported in the indicated column groups

Coefficient values *	Grouping factors			
	97.8 kDa	47.8 kDa	39.0 kDa	Total amount
A	-45.56	-314.6	-357.5	-717.7
B	3.837	11.22	13.86	28.92
C	-0.02868	-0.063554	-0.09868	-0.1909
Standard deviations				
A	5.313	7.133	4.678	14.74
B	0.1673	0.2246	0.1473	0.4641
C	0.001167	0.001567	0.001027	0.003237
R <sup>2</sup>	0.9918	0.9992	0.9994	0.9986

\* Referred to the 2<sup>nd</sup> order polynomial function  $Y = A + BX + CX^2$

## DISCUSSION

As widely reviewed by Reinmuth-Selzle et al. [25], atmospheric pollutants may have the following direct effects on pollen: (a) modifications to their biological and reproductive functions, (b) alteration of the physicochemical characteristics of the pollen surface, (c) change in the allergenic potential, and (d) adjuvant effect increasing their potential health hazards.

The present study evaluated the effect of outdoor atmospheric NO<sub>2</sub> gas pollutant on Cs pollen allergenic potential. Both proteic and allergenic patterns of Cs pollen extracts were in line with previous findings by Barletta et al [26], who reported that at least nine bands corresponding to gel-resolved Cs proteins were clearly bound by a rabbit antiserum against the Cs pollen extract. Interestingly, the same authors showed that components of about 100,

43 and 39 kDa molecular weight, respectively, were also recognized by human IgE of pooled Cs allergic sera. A similar immunoblotting IgE reactivity was also showed by Shahali et al. [27], particularly when Italian patients' sera were used, unlike French patients' sera that mainly recognized low molecular weight components. Although the minimal dose to induce the allergic sensitization or respiratory symptoms is unknown [28–30], in the current study it is assumed that increasing amounts of allergenic proteins in the pollen grains may represent a risk factor for allergic patients [31–33].

Data reported in this study indicate that in the city of Florence, the amount of pollen allergens can differ significantly, depending on the site where the Cs trees were located. Notably, the correlation model describing NO<sub>2</sub> air concentrations and Cs allergens expression relationship complies with a recent regulatory directive on non-linear models established by the United States Environmental Protection Agency [34]. Indeed, all four curves displayed a 2<sup>nd</sup> order polynomial trend, where the peak of allergens expression is reached at the intermediate value of mean annual NO<sub>2</sub> air concentration (67 µg/m<sup>3</sup>). Interestingly, this value is close to the concentration limit value for the protection of human health (40 µg/m<sup>3</sup>) (European Union Directive 2008/50/EC). This evidence suggests that 67 µg/m<sup>3</sup> mean annual NO<sub>2</sub> concentration, an apparently low risk value, should be regarded instead as being additionally harmful for sensitive subjects in relation to allergic respiratory reaction risks. Investigating on the detailed mechanisms of NO<sub>2</sub> modulation is beyond the scope of this study. However, it might be speculated that there are similar to that indicated by Zhao et al. [16], NO<sub>2</sub> gas behaves like a reactive nitrogen species that induce stress-related change in allergen genes expression.

It is important to consider the real allergenic content of pollen as a key parameter to be evaluated. With this in mind, it will be possible to shed new light on the issues of both pollen allergens exposure peak and clinical thresholds [35]. The importance of quantitative and qualitative determination of the seasonal allergen exposure peak has been underlined in a recent EAACI position paper [36], with the aim of correctly evaluating the efficacy of clinical trials for specific immunotherapy, as required by European Medicines Agency directives (CHMP/EWP/18504/2006).

## CONCLUSIONS

The study shows that intra-urban NO<sub>2</sub> variations do affect the expression of Cs pollen major allergens. The findings emphasize the need for a constant monitoring of the pollen allergens amount in parallel with assessment of the chemical pollutant levels. This will reflect on more comprehensive air quality policies, particularly within urban areas with increased motor vehicle density.



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