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# Year-to-year variation of the elemental and allergenic contents of *Ailanthus altissima* pollen grains: an allergomic study

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**Abstracts** The *Ailanthus altissima* pollen (AAP) has been reported as an emerging aeroallergen worldwide. This paper aims at examining the allergen pattern and the elemental composition of *A. altissima* pollen collected during two consecutive seasons (2014 and 2015). A gel-based allergomic study and SEM coupled to energy-dispersive X-ray (EDX) analysis have been carried out in order to evaluate the allergenic and elemental composition of AAP in two consecutive years. The IgE reactive patterns of 2014 and 2015 AAP PBS extracts were compared using the serum of a 31-year-old woman suffering from severe pollinosis symptoms to AAP. The EDX analysis revealed an important year-to-year variation in the ratios of some polluting elements such as nickel, sulfur, aluminum, lead, and copper. Gel alignments and comparative immunoproteomic analyses showed differential protein expression and IgE reactive patterns between AAPs collected in 2014 and 2015 pollinating seasons. From 20 distinct IgE-reactive spots detected in AAP extracts, 13 proteins showed higher expression in 2014 sample, while 7 allergen candidates exhibited an increased expression in AAP collected in 2015. Matrix-assisted laser desorption ionization-MS/MS analyses led to the identification of 13 IgE-binding proteins with confidence, all belonging to well-known allergenic protein families, i.e., enolase, calreticulin, and pectate lyase. Overall, the 2014 AAP showed higher concentrations of urban polluting elements as well as an increased expression of allergenic pectate lyase isoforms of about 52 kDa. This study demonstrates that the implementation of allergomic tools for the safety assessment of newly introduced and invasive plant species would help to the comprehensive monitoring of proteomic and transcriptomic alterations involving environmental allergens.

**Keywords** *Ailanthus* · Pollen · Heavy metal · Allergy · EDX · 2D electrophoresis

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## Introduction

IgE immunoproteomics referred to as "allergomics" has accelerated the identification of allergens from various sensitizing sources and has established precious links between sequences, structures, and immunological properties of allergenic molecules. The large amount of data generated by allergomic studies has given rise to multiple databases of allergenic proteins that today constitute a solid basis for progresses in molecular allergology and initiate a shift to molecular level in clinical practices and researches. Application of allergomics to safety assessment of invasive plants, particularly for those releasing high amounts of anemophilous pollen grains, could be of overwhelming importance to assess and control the risks of pollinosis in the general population. Allergomic studies can also provide precise information about the expression levels of allergens in various environmental conditions for invasive plants rapidly colonizing new areas.

*Ailanthus altissima* L. belongs to the Simaroubaceae family and represents a noxious invasive species in Middle East, Australia, New Zealand, the USA, and several countries in southern and central Europe. These trees are now present in all continents except Antarctica and pose an important risk for biodiversity, economies, and human health worldwide (Sladonja et al. 2015). Thanks to its good adaptation to the dry climate and different types of soils, the tree of heaven has been widely planted as an ornamental tree in urban areas in many cities of Iran. This fact increases the risks of interaction between pollen and environmental contaminants such as air pollution impacting many Iranian cities.

*A. altissima* pollen (AAP) has been reported as a source of allergens in many parts of the world (the USA, Italy, and recently in Iran) and its importance as an emergent allergen has been repeatedly emphasized (Blumstein 1943; Ballero et al. 2003; Vovolis et al. 2014; Maxia and Maxia 2003; Fereidouni et al. 2009; Majd et al. 2013; Mousavi et al. 2016). Recently, several allergen candidates such as enolase and pectate lyase have been identified from AAP (Mousavi et al. 2017). It has been previously shown that different environmental conditions can lead to an altered allergenic potential of pollen grains (Ghiani et al. 2016; Chassard et al. 2015). For instance, different geographic locations and times in season considerably influenced birch and grass pollen allergen contents (Buters et al. 2012 and Buters et al. 2015). Besides, pollutants can affect the expression of allergenic proteins in pollen (Shahali et al. 2009a; Chehregani et al. 2004; Majd et al. 2004; Suárez-Cervera et al. 2008; Rezanejad 2009). In addition, post-translation modifications induced by environmental factors may also alter allergenic properties of pollen allergens (Gruijthuijsen et al. 2006). Here, we have compared the elemental, protein, and allergen composition of *A. altissima* pollen grains collected in two consecutive years (2014 and 2015) from a male tree planted in an urban green space of Tehran, Iran. Finally, an allergomic approach has been used to evaluate the year-to-year variation of the allergenic content of *A. altissima* pollen grains.

## Materials and methods

### Allergic patient

A 31-year-old woman had pollinosis symptoms, including breathlessness, conjunctivitis, rhinitis, dry coughing, itching, and contact dermatitis, after direct exposure to AAP. According to our clinical analyses, the patient had a normal level of serum total IgE (33.2 IU/m) and was polysensitized to several inhalant allergens. Blood samples were drawn after obtaining an informed consent and stored at − 20 °C.

### Pollen sampling and protein extraction

Fresh pollens were collected during the late AAP season in 2014 and 2015 from a male tree planted in an urban green space of Tehran, Iran. Collected pollen grains were then sieved through an appropriate mesh (50 μm) to reach 99% purity. Pollen samples were then stored at − 20 °C until use. It is of great importance that the comparative studies be conducted on samples which have been stored and prepared in comparable conditions (e.g., storage conditions, extraction protocol). In this regard, pollen grains were collected from the same male tree under the same conditions (maturation level of pollens checked by electron microscopy) and using the same protocols for storage and protein extraction prior to analysis. Fifty milligrams (1/20 wt/vol) of defatted AAP was incubated under stirring in 1 mL phosphate buffer saline (PBS) pH 7.4 at 4 °C overnight. The extract was clarified by centrifugation at 12,000×*g* for 30 min at 4 °C and desalted by dialyzing against distilled water

overnight. Pollen extracts have been stored as aliquots at − 20 °C until molecular analyses.

### SEM/EDX analysis

SEM/EDX technique was used for the characterization of the elemental composition on pollen surfaces in both 2014 and 2015 samples. Briefly, the pollen samples were mounted on electron microprobe stubs and analyzed through a computer controlled field emission scanning electron microscope (SEM, Phillips XL30) equipped with an EDS-detector (EDS, Seron Technology AIS 2300C, Korea).

### Two-DE and western blot

The analyses on AAP extracts were performed using 2D-gel electrophoresis as previously described (Mousavi et al. 2017; Shahali et al. 2019). Briefly, samples were loaded onto immobilized gradient (IPG) strips, 7 cm pH 3–11 NL (GE Healthcare, Uppsala, Sweden) for the first-dimension run. The strips were focused using an EttanIPGphor 3 system (GE Healthcare) according to the manufacturer's instructions. After IEF, the strips were equilibrated for 20 min in equilibration buffers 1 and 2 containing dithiothreitol and iodoacetamide, respectively. In the second dimension, proteins were separated by laying strips on 12% SDS-PAGE gels. Two-D gels were run in duplicate and electro-transferred onto a PVDF membrane or stained with CBB-G-250. The molecular mass of protein bands was estimated using protein markers of known molecular weight (Fermentas, St. Leon-Rot, Germany). PVDF membranes were blocked and incubated overnight with the serum of the AAP allergic patient at 4 °C under shaking. After incubation for 1 h with alkaline phosphatase (AP)-conjugated goat anti-human IgE (Sigma-Aldrich, St. Louis, MO) and several washing, the membranes were treated with5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich, St. Louis, MO).

### Mass spectrometry and database searching

The IgE reactive spots have been subjected to MS/MS analysis using matrix-assisted laser desorption ionization time of flight (MALDI-TOF)/TOF mass spectrometry. Briefly, selected spots were excised from 2D gels, destained, and digested at 37 °C overnight with modified porcine trypsin (Promega, Madison, USA). Positive-ion MALDI mass spectra were obtained using a MALDI-TOF/TOF instrument (Bruker Ultraflex III, Elmsford, New York) in reflectron mode. The spectra generated were mass calibrated and deisotoped.

Tandem mass spectral data were submitted to database searching (NCBI nr database) using a locally running copy of the Mascot program (Matrix Science Ltd., version 2.4), through the Bruker Protein Scape interface (version 2.1). Results were filtered to accept only peptides with an expect score of 0.05 or lower.

### Gel alignments and comparative analysis

The 2D protein profiles of 2014 and 2015 AAP extracts were curated and analyzed using the Melanie 9-2D Gel Analysis Software (GE Healthcare, IL, USA), initially developed by the Swiss Institute of Bioinformatics (SIB). Melanie 9 has been used for the detection, quantification and matching of 2D gels for precise comparison of protein spots. In addition, the expression profile of IgE-reactive protein has been compared and assessed through statistical reports using standard *t* test and ANOVA statistics on relevant allergen candidates.

## Results and discussion

The planting of non-native plant species may lead to the emergence of new sensitizations in the population (Belmonte and Vilà 2004; Cariñanos and Casares-Porcel 2011). Gel-based immunoproteomics is now considered as the method of choice for identifying emerging allergenic sources (Mousavi et al. 2017; Shahali et al. 2014) and for studying the biochemical responses of allergenic plants to environmental stimuli (Schiavoni et al. 2017). In this report, the allergenic content of *A. altissima* pollen grains collected in 2014 and 2015 was analyzed using a two-dimensional gel electrophoresis (2-DE) proteomic approach and confronted to the serum of an allergic patient suffering from *A. altissima* pollinosis (Fig. 2a, b). As illustrated by the Fig. 1a, the AAP proteome was appropriately resolved using 2D gel electrophoresis and approximately 125 protein spots within the isoelectric point (pI) range of 3–11 and the MW range of 10–170 kDa were visible. Two-D immunoblotting of APP extracts probed with the serum of an AAP allergic patient revealed

several IgE-binding spots with MW and pI ranging from 20 to 55 kDa and 3.5 to 8.2, respectively (Fig. 1b). As depicted in Figs. 1 and 2, we detected 20 distinct IgE-reactive spots that showed consistent differences in expression levels between AAP collected in 2014 and 2015 (Fig. 3; Table 1). Thirteen of these proteins showed higher expression in the AAP collected in 2014, while seven allergen candidates exhibited an increased expression in the 2015 sample. The spots 4, 5, and 6 showed the greatest decrease in expression in 2015 (more than 2.5-fold). These 20 spots were selected for spot picking and trypsin digestion, and 13 of them were identified with confidence by MALDI-MS/MS and peptide mass fingerprinting (Table 1). Control experiments with the sera of healthy volunteers showed no reactivity with any spots (data not shown). Five

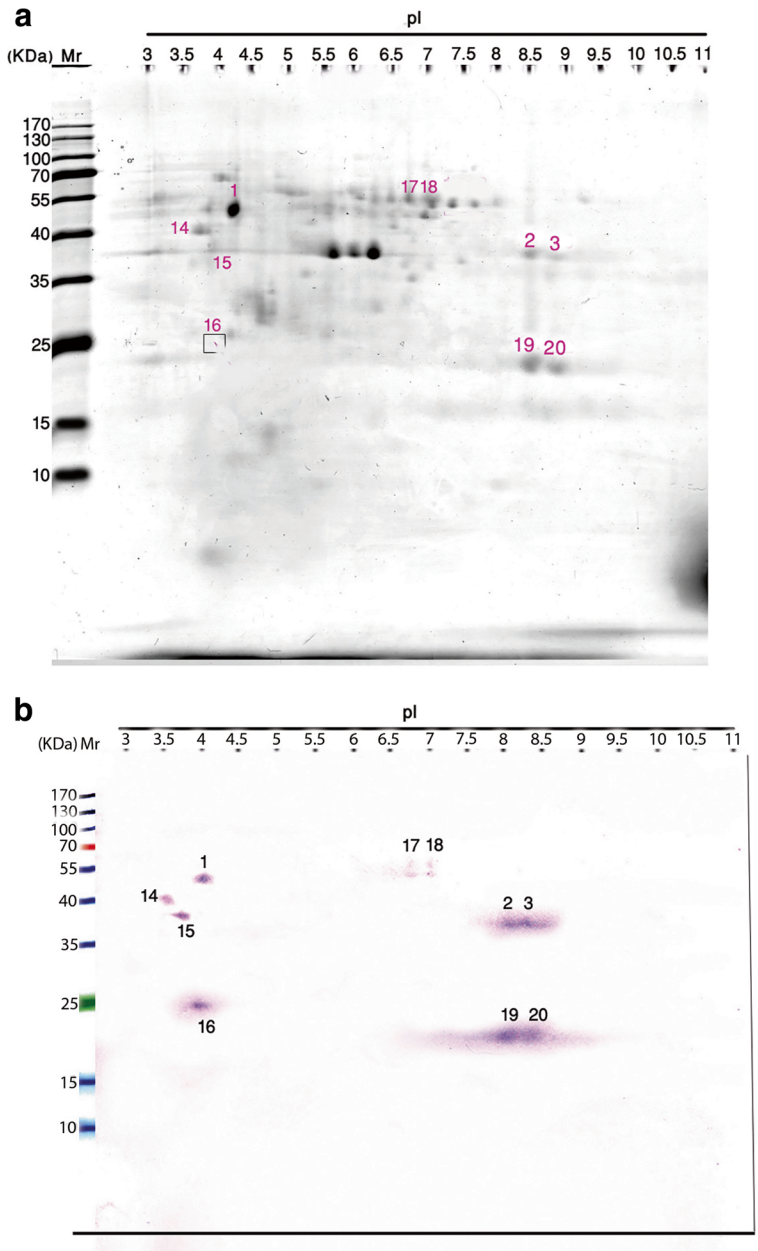


**Fig. 1** Two-DE and IgE immunoblot using 2015 AAP extracts. **a** Coomassie-stained 2-DE gel separation of a PBS extract from AAP collected in 2015. **b** Two-DE IgE immunoblot probed with the serum of a patient showing severe allergic reactions to AAP

allergenic proteins including enolase, calreticulin, enolase 2, probable pectate lyase 6, and pectate lyase were identified. MS/MS-based Mascot search using NCBInr database was statistically significant and identified all protein spots with relevant Mascot scores (Table 1). The spots 15–18 remained unidentified.

Besides the differences observed between the 2-DE protein profiles of AAP extracts collected in 2014 and 2015 (Figs. 1a and 2a), the present study has also revealed significant discrepancies in the allergen content of 2014 and 2015 pollen extracts (Figs. 1b and 2b). Interestingly, a direct relation has been observed

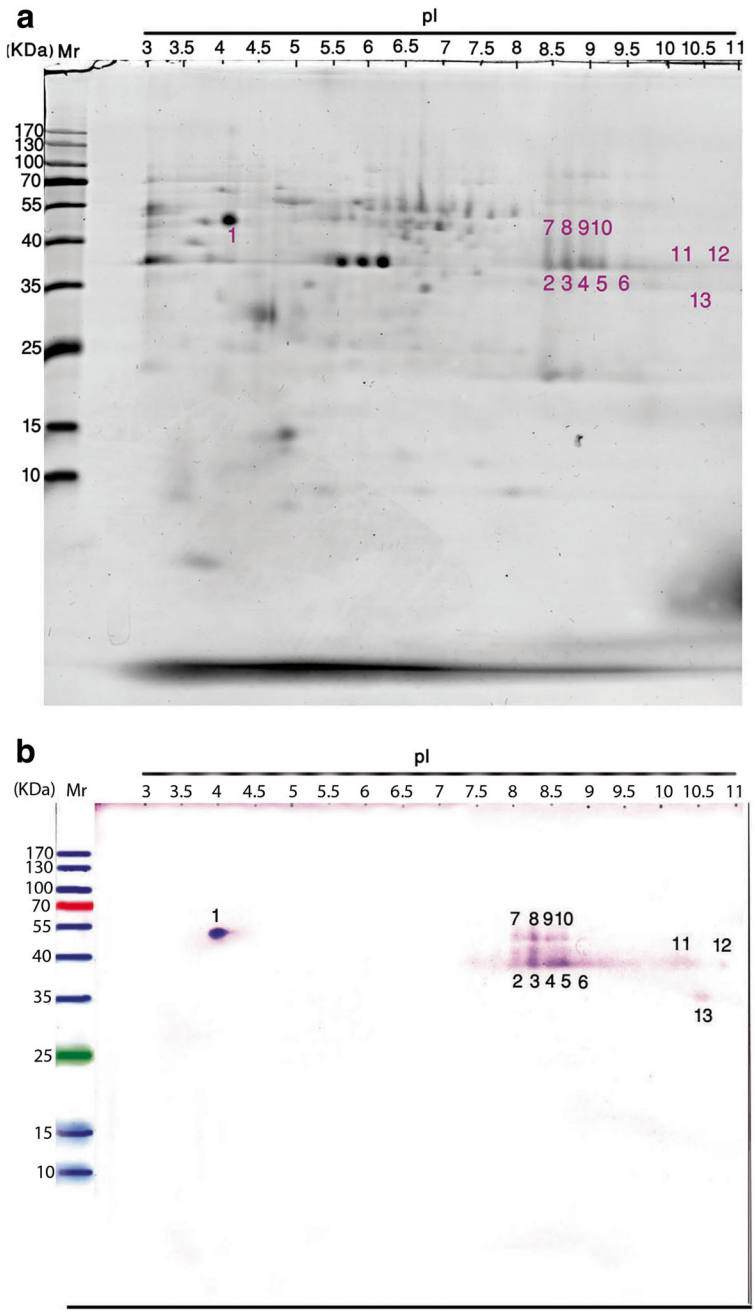


**Fig. 2** Two-DE and IgE immunoblot using 2014 AAP extracts (Mousavi et al. 2017). **a** Coomassie-stained 2-DE gel separation of the AAP PBS extract. **b** Two-DE IgE immunoblot probed with the serum of a patient showing severe allergic reactions to AAP

between the expression level and specific IgE recognition of candidate allergens (Fig. 3).

For example, the pectate lyase (PL) isoforms of about 52 kDa appeared to be differentially expressed in AAP collected in 2014 and 2015. The spots 2–10, which were homologous to PL, showed IgE-binding properties in 2014 AAP, whereas only two isoforms of these proteins (spots 2 and 3) were recognized by specific IgE in 2015 AAP. These results are probably due to the increased expression of the 52 kDa allergenic isoforms homologuous to PL in 2014 AAP as shown by the expression profiles provided by the Melanie 9 software (Fig. 3c). As illustrated by the Venn diagram presented in the Fig. 4, five other pollen proteins (spots 14–18) appeared to be IgE reactive only in 2015 AAP, while protein spot numbers 11–13 were only recognized by serum-specific IgE in 2014 AAP.

SEM/EDX analysis on 2014 and 2015 samples revealed the presence of magnesium(Mg), aluminum (Al), silica (Si), sulfur (S), chloride (Cl), potassium (K), calcium (Ca), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), silver (Ag), and lead (Pb) elements in different amounts. Values of urban polluting elements (Al, S, Fe, Co, Ni, Cu, Zn, Ag, and Pb) deposited on the pollen grains surfaces were significantly higher in 2014 samples (Table 2). Some bio-elements in pollen exist naturally in the amount of about 1.6%, including macronutrients (calcium, phosphorus, magnesium, sodium, and potassium) and micronutrients (iron, copper, zinc, manganese, silicon, and selenium). However, the values obtained for Mg and Cl were considerably higher in 2014. This may be directly related to different humidity levels in these two consecutive years, since the study of Duque et al. (2013) revealed a positive correlation between relative humidity

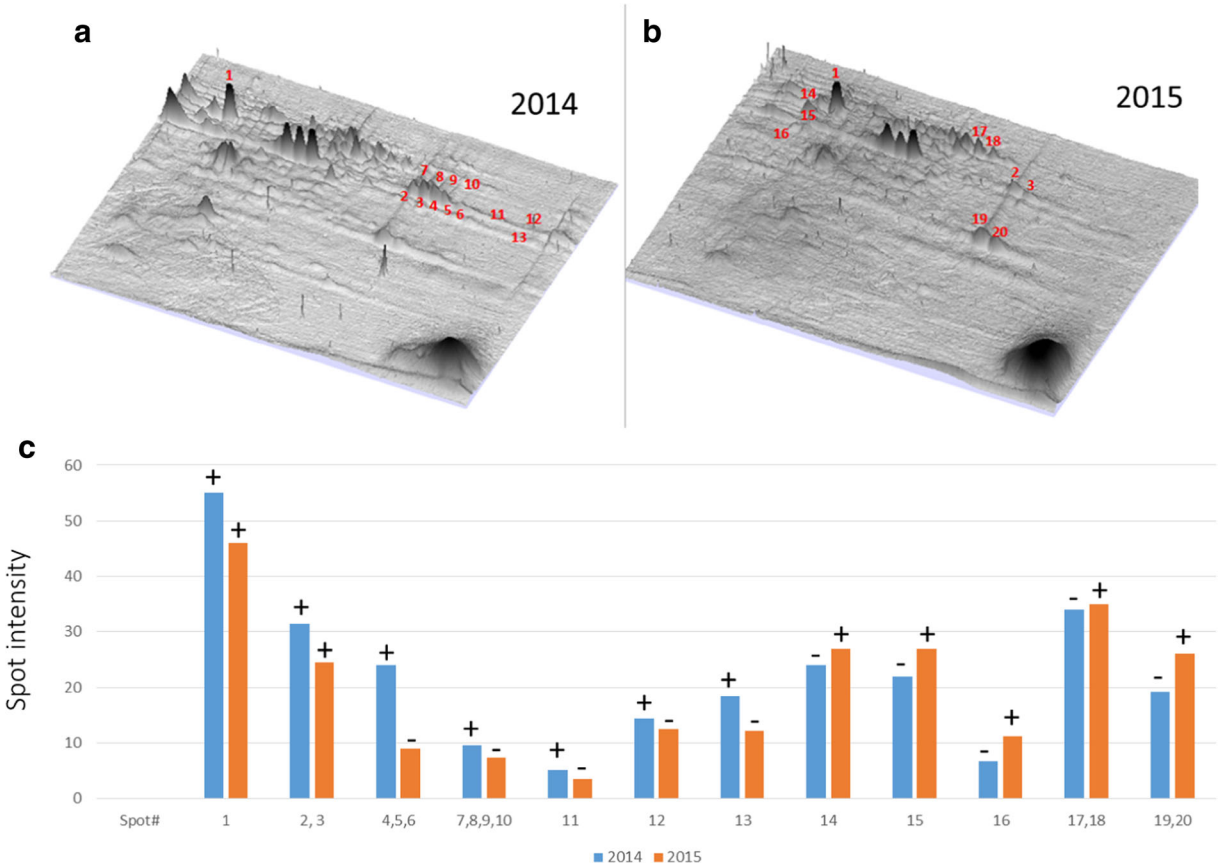


**Fig. 3** Expression profiles of IgE-binding protein spots detected from 2014 and 2015 pollen extracts. Gel alignments and comparative densitometry analyses between 2D protein patterns of 2014 and 2015 AAP (**a**, **b**) revealed differential expression profiles for the 20 IgE-binding proteins. **c** IgE reactive spots in 2014 and 2015 AAP are indicated by a positive sign

**Table 1** IgE reactive proteins identified from AAP extracts in 2014 and 2015 by MALDI TOF/TOF

| Spot | Accession number (NCBI) | Protein | Taxonomy | Peptide sequence | Cov (%) | Score | Mw/PI | 2014 AAP extract | 2015 AAP extract |
|---|---|---|---|---|---|---|---|---|---|
| 1 | gi\|158144895 | Enolase | *Gossypium hirsutum* | R.IEEELGAE/AV YAGASFR.A | 3 | 136 | 47.982/5.49 | ✓ | ✓ |
| | gi\|1009712 | Calreticulin | *Arabidopsis thaliana* | R.FYAISAEFPEF SNK.D | 3 | 9 | 46.782/4.37 | | |
| 2–6 | gi\|685325501 | Pectate lyase 6 probable | *Brassica rapa* | K.LADCVLGFGR. K/K.NPTIISQGNR.F | 4 | 130 | 52.041/9.82 | ✓ | Spots 2, 3 |
| 7–10 | gi\|685325501 | Pectate lyase 6 probable | *Brassica rapa* | K.LADCVLGFGR. K/ K.NPTIISQGNR.F | 4 | 125 | 52.041/9.82 | ✓ | ✘ |
| | gi\|223542934 | Conserved hypothetical protein | *Ricinus communis* | K.QGMADALAEM TKR.S + oxidation (M) | 2 | 56 | 64.915/6.64 | | |
| | gi\|743851961 | Ras-related protein RHN1-like | *Elaeis guineensis* | K.QGNSNMVTALAGNK.A + 3 deamidated (NQ); oxidation (M) | 6 | 55 | 22.195/5.78 | | |
| 11–13* | | | | | | | | ✓ | ✘ |
| 14 | gi\|728841144 | Enolase 2 | *Gossypium arboretum* | R.IEEELGAEAVYA GASFR.T | 3 | 137 | 46.895/5.98 | ✘ | ✓ |
| 15–18* | | | | | | | | ✘ | ✓ |
| 19, 20 | gi\|475603422 | Pectate lyase | *Aegilops tauschii* | K.NPTIISQGNR.Y | 3 | 73 | 36.732/8.53 | ✘ | ✓ |

*Non-identified protein

and the amount of Cl, Na, and Mg on the pollen surface of three different pollen grains. The same authors also reported that Cl mass percentages positively correlated with the amount of precipitation. In contrast, the level of Si was significantly higher in 2015 AAP. Si is a potential marker of mineral dust exposure under windy and dry weather conditions (Duque et al. 2013). Taken together, our comparative EDX analyses suggest that 2014 AAP were exposed to higher levels of urban pollution when compared to AAP collected in 2015 and it can as well be suggested that flowering season of *A. altissima* in 2014 was drier.

Heavy metal emissions from traffic and industrial activities are common pollutants within urban areas. Due to the porosity and the lipophilic structure of their outer wall (exine), pollen grains are considered as efficient bioindicators, which are able to absorb air pollutants such as heavy metals (Kalbande et al. 2008). The heavy metal ions can interact with DNA and nuclear proteins of pollen grain and cause changes in its genes expression (Tchounwou et al. 2012). Traffic-related air pollution affects directly the elemental composition of pollen grains (Shahali et al. 2009b). In our study, a significant increase in heavy element quantities adhering to the pollen grains surfaces was observed in 2014 compared to 2015. It may be possible to attribute the differential expression of some pollen allergens in 2014 to higher levels of polluting elements. An increased expression of pollen allergenic proteins has previously been reported in areas with severe pollution (Bartra et al. 2007). It has been previously found that pollen protein expression could be highly influenced by some environmental stressors such as

**Table 2** Elemental composition of AAP in 2014 and 2015 detected by EDX analysis (%)

| Elements | Mg | Al | Si | S | Cl | K | Ca | Fe | Co | Ni | Cu | Zn | Ag | Pb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2014 | 10.09 | 8.75 | 5.80 | 5.11 | 2.93 | 22.85 | 9.69 | 4.94 | 4.34 | 5.63 | 6.88 | 7.71 | 2.84 | 2.45 |
| 2015 | 2.00 | 1.42 | 7.51 | 2.53 | 1.35 | 12.90 | 64.58 | 2.87 | 0.46 | 0.61 | 0.61 | 0.75 | 0.48 | 1.94 |

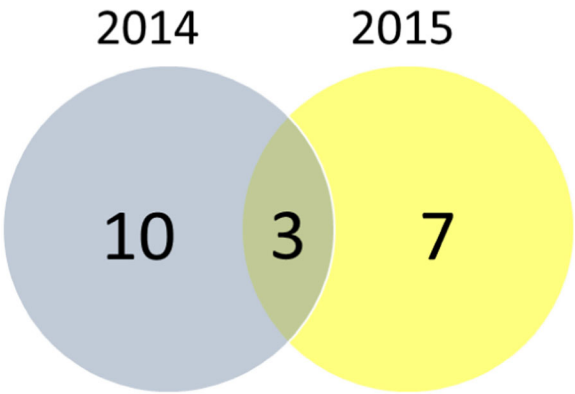


**Fig. 4** Overlapping Venn diagram illustrating exclusive and common IgE-binding protein spots detected from 2014 and 2015 AAP extracts

temperature, drought, and microbial infections as well as following UV, $NO_2$, and ozone exposure (Sénéchal et al. 2015). Other factors including shading, soil properties, and genetics appeared to have strong influences on the composition of birch pollen allergens (Helander et al. 1997). Pollutants play a significant role in altering the total protein content of pollen grains and may modify the protein profile and the expression of specific proteins in pollen extracts (Malayeri et al. 2012). In addition, it was found that some gaseous air pollutants such as NOx and ozone are able to affect allergenic proteins by nitration (Liu et al. 2017). These chemical modifications affect the allergenic potency of airborne pollen grains (Karle et al. 2012) and may even lead to the formation of new epitopes (neo-epitopes) in some allergens such as Bet v 1 (Gruijthuijsen et al. 2006). Environmental factors may also considerably impact the content in major allergens of pollen grains. Shahali et al. (2009a) reported a reduction in the total protein content of *Cupressus arizonica* pollen exposed to the polluted environments of Tehran, as well as a drastic decrease of the Cup a 1 major allergen (Shahali et al. 2009a; Sénéchal et al. 2015). In contrary, Lu et al. (2014) using two-dimensional electrophoresis revealed a clear increase in the *Platanus* pollen major allergen (Pla a 1) with a molecular weight of 18 kDa, following exposure to gaseous pollutants. Ghiani et al. (2016) also reported the increased allergenicity of pollen grains released from ragweed plants along polluted and heavy traffic roads. Another study has also reported that the content of Amb a 1, the ragweed pollen pectate lyase, varied from one place to another and from year-to-year (Lee and Yang 1979; Shea et al. 2008). This is in accordance with the results of the present study, showing a 2.5-fold increase in the expression of pectate lyase allergenic isoforms (spots 2–10) in *A. altissima* pollen carrying higher concentrations of urban polluting elements.

## Conclusion

In conclusion, the results of the present study revealed differences in the elemental, protein expression, and allergen composition of AAP collected in two consecutive years. This can be due to differential growth and environmental conditions considering the fact that 2014 AAP showed higher concentrations of urban polluting elements on their pollen wall surfaces. These results stress the need to implement new tools in order to better evaluate proteomic and transcriptomic alterations involving pollen allergens. For emerging allergens such as AAP, translational laboratory experiments and comprehensive monitoring networks are required to assess allergenic risks of *A. altissima* plantation as well as its invasive expansion in urban green spaces of many regions worldwide.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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