

Effect of environmental pollution on the proteins, allergenic bands, ontogeny and structure of *Avicennia marina* (Forsk.) Vierh (Avicenniaceae) pollen grains

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Abstract In Bushehr province of Iran, *Avicennia marina* trees have grown in Bordekhoon (Mond Protected Area) and Assaluyeh (Marine National Park of Nayband). Contrary to Bordekhoon, Assaluyeh is a petrochemical region with environmental pollution. This study was aimed to studying protein profiles, allergenic bands, ontogeny, structure and elemental composition of tectum of *A. marina* pollens in Assaluyeh and Bordekhoon. Pollens were collected from two regions and extracted in PBS, and protein profiles of pollens were determined by sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). As an experimental model, 20 female 6–8-week-old Balb/C mice were divided into two groups. The mice of first and second groups were sensitized by Bordekhoon and

Assaluyeh pollen extracts, respectively, and mice serum samples were used for immunoblotting. Pollen characteristics were studied using light and scanning electron microscopes. SDS-PAGE showed some differences between pollen protein profiles of two regions. Immunoblotting assay detected that pollens have two allergenic bands and the protein band at 100 KD is the common allergenic protein in two regions. Light microscopy revealed that the development of anther wall was basic type and some abnormalities were observed in microspores and pollens of Assaluyeh. Scanning electron microscopy studies showed that the apertures in considerable numbers of Assaluyeh pollens were closed. The comparison of elemental composition of pollen tectum between two regions showed that pollens of Assaluyeh have accumulated Cu on their tectum. Results obtained indicated that environmental pollution can affect protein profile, allergenic bands, structure and elemental composition of tectum of *A. marina* pollens.

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1 Introduction

The family of Avicenniaceae belongs to the order Lamiales (Borg and Schonenberger 2011). This family

comprises of only one genus *Avicennia* (Ghosh et al. 2008). The mangrove plants are characteristic of tropical and subtropical coastlines (Sun et al. 1998), and the gray mangrove *Avicennia marina* (Forsk.) Vierh is widespread on the coasts of Asia and Australia (Clarke and Myerscough 1991). In Bushehr province of Iran and on the coasts of Persian Gulf, *A. marina* trees have grown in two regions: Assaluyeh (Marine National Park of Nayband) and Bordekhoon (Mond Protected Area). Assaluyeh is an industrial city and is regarded as Iran's main economic base and the world's largest energy production (Jozi and Salati 2012). Some factors threaten mangrove ecosystem in Assaluyeh such as air pollution (Salehi et al. 2012) and oil pollution as a result of development of the southern pars oil field and waste water that discharges to this area and is mainly composed of heavy metals (Zahed et al. 2010).

Pollen grains transport male gametophytes in angiosperms (Irian et al. 2012). They are one of the bases for correct fertilization, and each factor that affects them may influence seed production that will produce the next generation (Rezanejad 2007; Qu et al. 2010). Pollens from some species count with allergens, and it is important to pay attention to anemophilous plants (Jianan et al. 2007). Pollen allergens are organic materials that have been located in the pollens and their walls (Majd and Ghanati 1995). Several materials in pollen grains have mentioned as sources of allergy. March et al. (1970) proposed that polysaccharides are pollen allergens, or Ramirez (1984) introduced glycoproteins as allergen materials in pollen grains. Haavic and Smestad Paulsen (1985) and Jarolim and Matthiesen (1990) in their studies reported that basic and acidic proteins and polypeptidic groups act as pollen allergens. There are several reports that air pollution enhances allergenic characteristics of pollen grains (Shahali et al. 2009; Armentia et al. 2002). Salehi et al. (2012) who worked on the allergenicity of *A. marina* pollens reported that some allergenic reactions such as total serum IgE level in mice treated with Assaluyeh (polluted area) pollen extract were significantly higher than the mice treated with Bordekhoon (control area) pollen extract.

Avicennia marina is one of the major vegetative covers of Bushehr province, which has wind scattered pollens (Salehi et al. 2012). Our previous work showed that *A. marina* pollens have allergenic properties and air pollution intensifies these properties (Salehi et al.

2012). Therefore, one of the aims of the present study was to detect the pollen allergenic bands in control and polluted regions. Comparing protein profiles and development of polluted and control areas pollens was another objective of this paper to investigate the effects of air pollution on ontogeny and proteins of pollen grains.

2 Materials and methods

2.1 Study areas and climate

Assaluyeh and Bordekhoon are coastal regions that are situated adjacent to the Persian Gulf (Fig. 1) (Nouri et al. 2007; Azarmsa, 2008). Assaluyeh is a petrochemical region, which has a lot of gas and oil production factories and refineries that pollute the area, and these factories do not exist in Bordekhoon. In this study, Assaluyeh is assumed as polluted area and Bordekhoon as control area. These two regions have hot and humid climate (Nouri et al. 2007; Azarmsa 2008). Bordekhoon is situated between 27°15' and 28°45' north latitude and 51°15' and 51°35' east longitudes with a mean annual humidity and raining of 70 % and 225 mm, respectively (Zare-Zadeh Mehrizi et al. 2011). Assaluyeh is situated between 27°28'15" and 27°92'5" north latitude and 52°27'28" and 52°52'20" east longitude with the average annual humidity and raining of 64 % and 193 mm, respectively (Zare-Zadeh Mehrizi et al. 2011). The distance between these two regions is about 160 km.

2.2 Pollen sampling

During spring and summer 2011 (June–July), the pollens were collected from flowers of *A. marina* trees grown in control (Bordekhoon) and polluted (Assaluyeh) areas. Some of the collected pollens were dried for scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analysis, and some of them were stored at −20 °C for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Storage was the same in two regions.

2.3 Pollen extract preparation

For preparation of pollen extracts, 50 mg of pollens of each samples was extracted in 1 ml of phosphate-

Fig. 1 Location of the study area



buffered saline solution (PBS) pH = 7.4 (1:20). The mixture was stirred overnight at 4 °C. The suspension was centrifuged at 4,600 rpm for 45 min, and supernatants were dialyzed against distilled water (Salehi et al. 2012).

2.4 Protein studies

Loading buffer (0.125 M Tris–HCl pH 6.8; 20 % glycerol; 4 % sodium dodecyl sulfate; 0.1 % bromophenol blue; 10 % β -mercaptoethanol) was added to 90 μ l of control and polluted areas pollen extracts (including 10 μ g protein) and after vortexing and heating in boiling water for 3 min were separated by means of 12 % SDS-PAGE (Laemmli 1970). The separated proteins were detected by Coomassie blue staining and then destained with destaining solution (methanol/acetic acid/water in a 1:1:8 ratio). The total protein contents of control and polluted areas pollen

extracts were estimated as explained in the Bradford protein assay using bovine serum albumin as standard (Bradford 1976).

2.5 Animals and sensitization

Twenty female 6–8-week-old Balb/c mice were obtained from Pasteur Institute (Karaj, Iran). Animals were maintained at the animal care service of the Tehran University of Medical Sciences. They were divided into two groups ($n = 10$). The mice of first group were treated with 180 μ l pollen extract (including 20 μ g protein) of control area adsorbed to 2 mg of $\text{Al}(\text{OH})_3$ (Sigma Chemical Co.) as an adjuvant. The mice of second group were treated with polluted area pollen extract in the same route. All injections were performed intraperitoneally on days 0, 7, 14 and 21 (Barral et al. 2006). Mice were anaesthetized with ether, and blood samples were collected from the heart

on day 28. Serum samples were prepared and stored at -20°C for immunoblotting.

2.6 Immunoblotting assay

Following electrophoresis, proteins were transferred from the gel on to polyvinylidene difluoride (PVDF) membrane. After blocking the membrane in skim milk 5 % overnight at 4°C , the membrane was washed three times for 5 min in TBS-Tween 20 and incubated at room temperature for 2 h with 1:100 diluted sera from sensitized mice. Then, the membrane was washed three times for five min. Bound mouse IgE was detected with 1:5,000 goat anti-mouse IgE Horse radish peroxidase (Koma Co., Seoul, Korea). The membrane was washed four times for 10 min and then treated by ECL chemiluminescence according to the manufacturer's instructions (Amersham kit, Buckinghamshire, England). The allergen bands were revealed after 30-s contact with Kodak film (Towbin et al. 1979).

2.7 Light microscopy

Flower buds in different developmental stages and mature flowers of *A. marina* trees were collected from control and polluted areas, fixed in FAA (formalin %37/glacial acetic acid/ethanol 96° in a 2:1:17 ratio) for 12 h, washed with ddH₂O, dehydrated through graded ethanol series and embedded in paraffin. The samples were sectioned at 8–12 μm using a rotary microtome, stained with hematoxylin eosine and studied by Zeiss light microscope. In each region, for at least 100 pollen grains, different kinds of irregularities were studied and the pollen regularity (%) was calculated in the control and polluted areas. This study was repeated for four times.

2.8 SEM and EDX studies

Dried pollens were coated with gold by physical vapor deposition (Sputter coater SCDOOS, BAL-TEC, CO, Switzerland) and then were observed by a scanning electron microscope (Philips XL 30). Apertures of 100 pollen grains were studied in each region, and the percentage of pollens with closed apertures was calculated. This procedure was repeated four times.

The composition of elements on pollens tectum of control and polluted areas was determined by using EDX analysis.

2.9 Statistical analysis

ANOVA and Duncan's test were used for statistical analysis.

3 Results

3.1 SDS-PAGE analysis

Sulfate–polyacrylamide gel electrophoresis analysis showed some differences in protein patterns and quantities of protein bands between two regions. The protein profiles of pollen samples revealed that protein bands at about 15 and 20 KD that were present in polluted area pollens did not exist in control area pollens, and interestingly, the protein bands at about 10 and 25 KD in control area pollens were not visible in polluted area pollens (Fig. 2). The density of protein band at 100 KD also increased in control area compared with polluted area pollen grains (Fig. 2).

3.2 Immunoblotting assay

In order to determine the allergenic proteins in pollens, immunoblotting with sera of mice treated by control and polluted areas pollen extracts and conjugated anti-IgE was carried out. It revealed that protein bands at

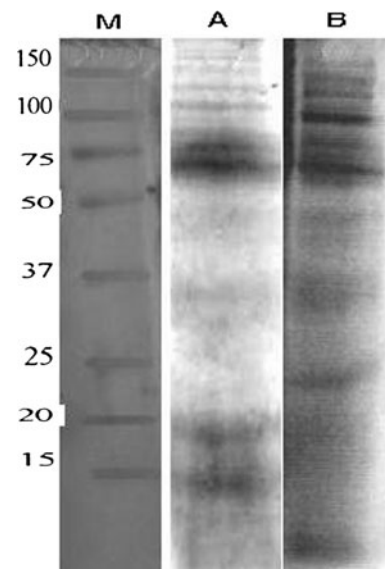


Fig. 2 Pollen protein profiles of *A. marina* grown in Assaluyeh (a) and Bordekhoo (b). marker (M). (Sizes are in KD.)

100 and 20 KD in polluted area and 100 and 25 KD in control area pollens were allergen proteins that reacted with the IgE antibodies induced in mice treated with pollen extracts of two regions (Fig. 3). Therefore, the protein band at 100 KD is the common allergen protein in pollens of two regions.

3.3 Anther wall and pollen ontogeny in control area

In *A. marina*, the young anther wall is composed of five layers from outer layer to inner layer: an epidermis, an endothecium, two middle layers and one layer of tapetum (Fig. 4a). In young anther wall, the endothecium is one-layered (Fig. 4a), but as the anther wall develops, endothecium cells divide and two layers of it with the cells that show longitudinal elongation and helical thickenings can be seen (Fig. 4b, i). At the early stages of microsporogenesis, the sporogenous tissue is surrounded by the tapetum. Sporogenous tissue is multilayered with the cells are placed close to each other (Fig. 4a). They have big nucleus and are origin of microspore mother cells with several mitotic divisions (Fig. 4a). Microspore mother cells are angular in shape (Fig. 4b), but gradually, they enlarge and round up (Figs. 4c and 5a). During the telophase I, microspore mother cells produce dyads (Figs. 4c and 5b). After this stage, tetrahedral tetrads form (Figs. 4c and 5c). Meanwhile, the cells of middle

layers enter to pollen sac and degenerate to supply nutrition for further development of microspores (Fig. 4d). During microspore development, the septum between two pollen sacs degenerates and two pollen sacs merge to form a single locule (Fig. 4d). At the late microsporogenesis, microspores are released from tetrads and the callose wall that has surrounded them and form free microspores (Fig. 5d). The young microspores have central nucleus, and the exine ornamentation is obvious (Fig. 4e). The mature microspores enlarge and round and have peripheral and big nucleus (Fig. 4g). Until early steps of young microspore stage no invasion of tapetum cells into locular space were occurred therefore till this stage the tapetum layer belongs to glandular type (Fig. 4e) but at the end of this stage tapetum layer enters locule and transforms to amoeboid type (Fig. 4f). In the mature microspore stage, the tapetum layer has entirely surrounded the microspores (Fig. 4g). So, the tapetum layer in this plant is dimorphic. Pollen grains, in size, are smaller than mature microspores and have two nuclei (Fig. 4h). In the mature anther, the tapetum cells have not completely degenerated and some of them have collected in the corner of the locule (Fig. 4j). In this stage, the anther wall is consisted of epidermis and endothecium (Fig. 4i).

3.4 Anther wall and pollen ontogeny in polluted area (Assaluyeh)

There were no significant differences in anther wall and pollen ontogeny between polluted and control areas, but abnormalities in microspores and pollens of polluted area increased. Some microspores showed irregular shapes and breakage of exine (Fig. 6a). In this region, some pollens were very small (Fig. 6b), some of them had very thick exine (Fig. 6c) and an amorphous mass was seen in some pollen sacs (Fig. 6d). Our results showed that pollen regularity (mean) was about 91 % in control area and 62 % in polluted area, but statistical analysis showed that this difference was not significant ($p > 0.05$).

3.5 SEM studies

Electronic micrographs obtained by SEM studies have shown in Fig. 7. Calculating the percentage of pollens with closed apertures showed that 93 % of polluted area pollens had closed apertures, but only in 1 % of

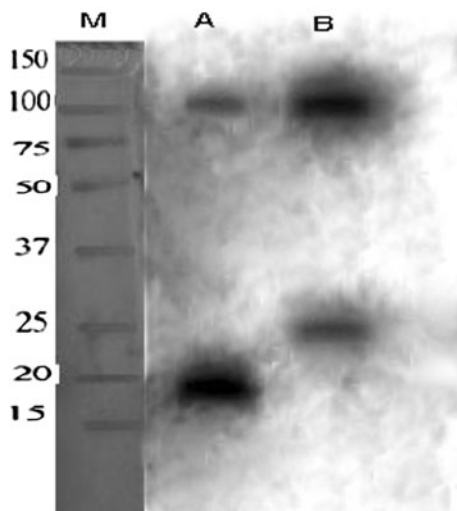


Fig. 3 Allergenic bands of *A. marina* pollens grown in Assaluyeh (a) and Bordekhoon (b) detected by immunoblotting method. Marker (M). (Sizes are in KD.)

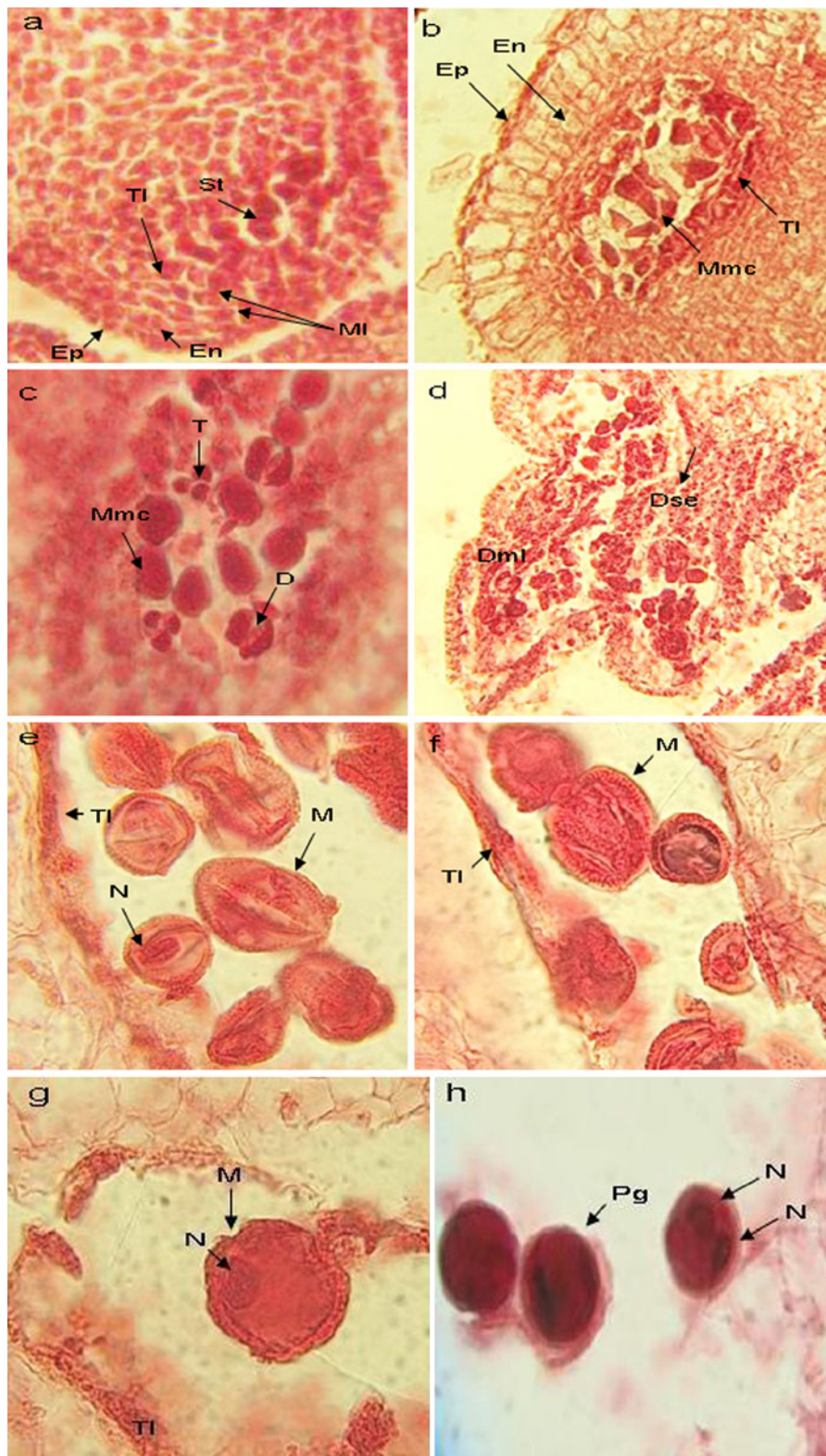


Fig. 4 Ontogeny of *A. marina* pollen grains grown in Bordekhoon (control area). **a** Young anther wall (mag. $\times 400$). **b** Microspore mother cells that are *angular* in shape (mag. $\times 400$). **c** Spherical microspore mother cells, dyads and tetrads (mag. $\times 1,000$). **d** Degenerated septum and middle layers (mag. $\times 100$). **e** Young microspores with central nucleus (mag. $\times 1,000$). **f** The tapetum layer enters the locule (mag. $\times 1,000$). **g** The tapetum layer surrounds each mature microspore (mag. $\times 1,000$). **h** Mature pollen grain with two nuclei (mag. $\times 1,000$). **i** Mature anther wall (mag. $\times 1,000$). **j** The tapetal cells that have collected in the corner of locule in mature anther (mag. $\times 400$). Dyad (*D*); degenerated middle layers (*Dml*); degenerated septum (*Dse*); endothecium (*En*); epiderm (*Ep*); helical thickenings (*Ht*); microspore (*M*); microspore mother cell (*Mmc*); middle layers (*ML*); nucleus (*N*); pollen grain (*Pg*); sporogenous tissue (*St*); tetrad (*T*); tapetum cells (*Tc*); tapetum layer (*TL*)

control area pollens the apertures were closed. Statistical analysis revealed that this difference was considerable ($p < 0.05$).

3.6 EDX studies

Energy-dispersive X-ray analysis determined that Na, Cl, K, Ca and Cu were essential elements that compose the tectum of *A. marina* pollens (Table 1). The comparison of elements between polluted and control areas revealed that the ratio of Cu (%) in polluted area was more than 17-fold higher compared with control area.

4 Discussion

Industrial pollutants that exist in the air interact with pollen grains and change their ontogeny, physiology and morphology (Rezanejad 2009). There are several

studies that indicate air pollution can intensify pollen allergenicity. Air pollutants by adhering to the allergenic pollen surface act as adjuvant and increase immune response to allergenic materials of pollens (Armentia et al. 2002). Salehi et al. 2012 showed that industrial pollution in Assaluyeh region affected some allergenic characteristics of *A. marina* pollens. They showed that wheal diameter of intradermal skin test, eosinophil rate in blood smears and total serum IgE level in mice treated with Assalueh (polluted area) pollen extract were higher than in the mice treated by Bordekhoon (control area) pollen extract. They showed that *A. marina* pollens have allergenic properties and air pollution can intensify these characteristics.

4.1 SDS-PAGE analysis

Our results showed that pollen protein profiles of control and polluted areas were different; thus, environmental pollution in Assaluyeh may be responsible for these differences. Similar to our results, Behrendt et al. (2001) and Armentia et al. (2002) have already observed the difference between pollen protein profiles of polluted and non-polluted areas; however, some researches did not observe any differences (Helander et al. 1997; Chehregani et al. 2004). Thus, kind of plant species and characteristics of pollens such as thickness of exine may be the reason for these contradictory results (Rezanejad 2009).

4.2 Immunoblotting assay

Immunoblotting assay indicated that the allergenic band at 100 KD was observed in two regions, but

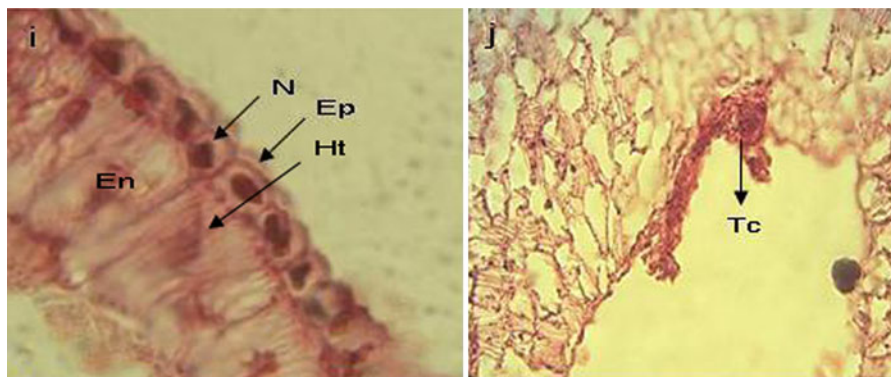


Fig. 4 continued

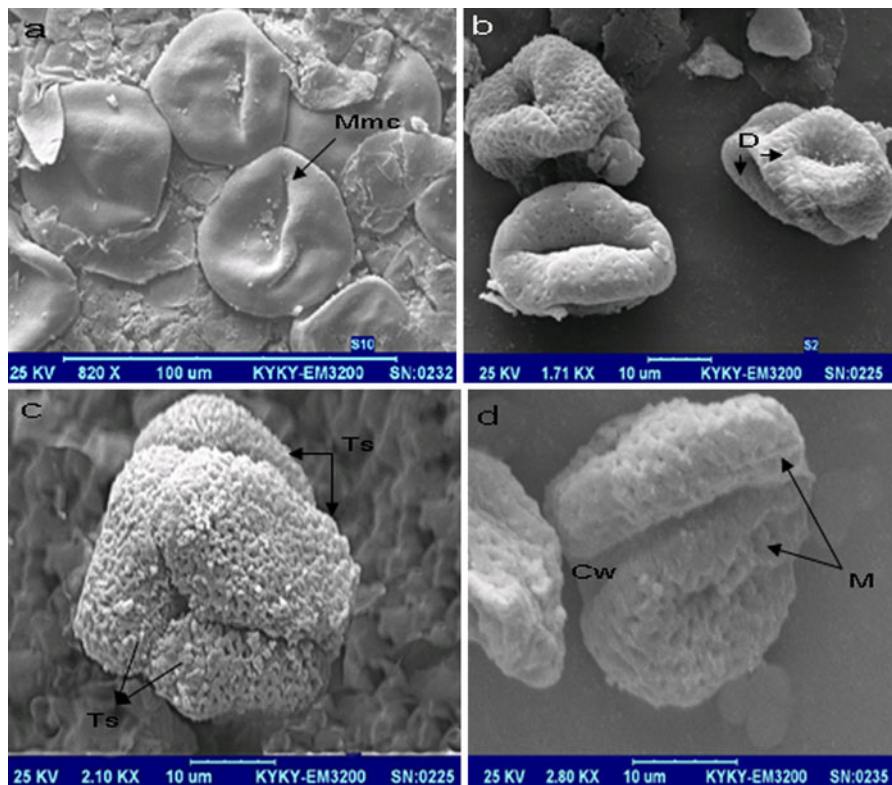


Fig. 5 Scanning electron micrographs of microspore mother cells (*Mmc*), dyad (*D*), tetrads (*Ts*) and microspores (*M*) that are releasing from each other and callose wall (*Cw*)

allergenic bands at 20 and 25 KD were observed in polluted area and control area, respectively. Similar to our results, Behrendt et al. (1997) observed air pollution changes allergenic band to lower molecular weight bands. However, some studies showed no difference between the allergenic bands of polluted and non-polluted areas (Helander et al. 1997). Maybe type of plant species causes these differences, but it needs to be studied future.

4.3 Anther wall and pollen ontogeny in control area

Based on our results, young anther wall in *A. marina* is composed of five layers. In some species of *Jatropha L.* and in *Vitis vinifera*, young anther wall is also consisted of five layers (Sharma and Rana 2010; Buyukkartal et al. 2005). In young anther wall, one layer of endothecium is observed, but as it develops, the layer of endothecium increases and shows helical thickenings. Consistent with our observations, helical thickenings of endothecium and increasing of its

layers during pollen wall development were seen among the species of *Heliconia* (Simao et al. 2007). Our studies showed that microspore mother cells during first and second stages of meiosis produce dyads and tetrads thus cytokinesis is not simultaneous. Magnard et al. (2001) showed that in angiosperms, cytokinesis is simultaneous; therefore, contrary to other angiosperms in this plant, the formation of wall between microspores occurs in first and second phases of meiotic division. During anther wall development, middle layers degenerate to produce nutrition for the development of microspores. Some authors showed that in Caryophyllaceae, Oleaceae and Leguminosae, the middle layers are ephemeral (Qu et al. 2010; Grancharova et al. 2011; Hardy et al. 2000). The septum between two pollen sacs gradually degenerates. This phenomenon in some species of *Heliconia* has been seen (Simao et al. 2007). The tapetum layer in *A. marina* is dimorphic. Generally, two major tapetum types exist in plants: glandular type and amoeboid (plasmodium) type (Oldenhof and Willemse 1999), but a third type can also be seen, this type

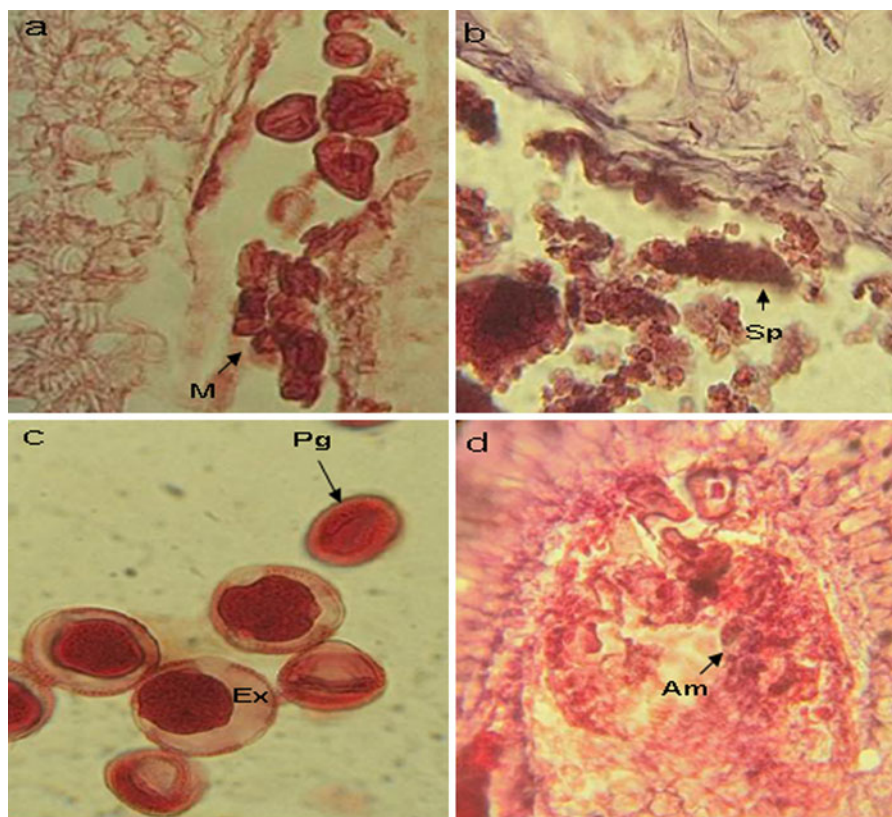


Fig. 6 Light micrograph of some abnormalities in microspores and pollen grains of Assaluyeh (polluted area). **a** Irregular microspores (mag. $\times 400$). **b** Small pollen grains (mag. $\times 400$).

c Pollen grains with thick exine (mag. $\times 1,000$). **d** An amorphous mass in pollen sac (mag. $\times 400$). Amorphous mass (*Am*); exine (*Ex*); microspore (*M*); pollen grain (*Pg*); small pollens (*Sp*)

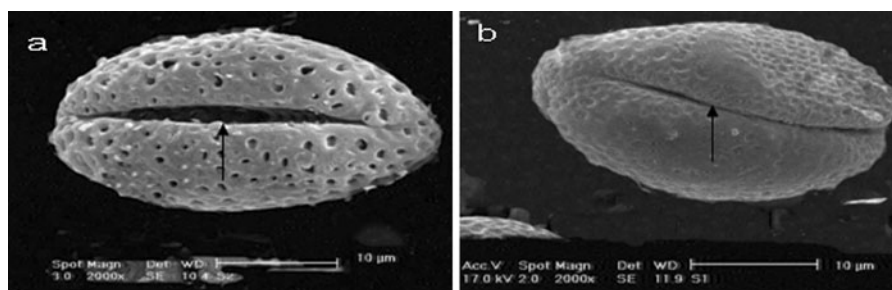


Fig. 7 Scanning electron micrographs of control (**a**) and polluted areas (**b**) pollen grains aperture (arrow)

is rare, but exists in some plants (Furness 2008). In this kind, after releasing microspores from tetrad, plasmodium forms and surrounds microspores (Furness 2008). Our results about tapetum type in *A. marina* are consistent with Furness (2008) that observed the third type of tapetum in some Asterids.

Table 1 Elemental composition (%) of pollen tectum of control and polluted areas

Elements	Na	Cl	K	Ca	Cu
Control area (%)	4.9	63.4	17.4	11.4	2.9
Polluted area (%)	3.55	31.48	7.84	7.32	49.79

4.4 Anther wall and pollen ontogeny in polluted area (Assaluyeh)

Our studies indicated that pollen irregularities increased in polluted area compared with control area; however, this increasing was not significant. Other authors showed that air pollutants can cause abnormalities in pollen shape (Majd and Mohammadi 1992; Pelter 1998; Emberlin 2000). They reported that air pollutants stick to the pollens surfaces and cause abnormalities in the shape of pollen grains.

4.5 SEM studies

Scanning electron microscopy studies showed that considerable numbers of polluted area pollen grains had closed apertures. This is in agreement with Rezanejad (2007) who observed the apertures of *Spartium junceum* pollens collected from polluted area were closed. Some researchers reported that airborne pollutants adhere to the pollens surfaces and cause abnormal pollens (Emberlin 2000; Pelter 1998). These structural abnormalities can induce pollen sterility (Rezanejad 2007).

4.6 EDX studies

Comparing elemental composition of pollen tectum between the two regions revealed that polluted area pollens have accumulated Cu on their tectum. Pollens can accumulate heavy metals such as Cu on their surfaces (Sawidis and Reiss 1995; Sawidis 1997; Kalbande et al. 2008). The roots of *Avicennia marina* absorb heavy metals such as Cu and Zn and transfer them to other organs, but other heavy metals such as Pb remain in the roots (Macfarlane and Burchet 2001). This can be the reason for the high concentration of Cu on pollen tectum.

5 Conclusions

The present study showed that pollen proteins and immunoblot profiles of control and polluted areas were different. Microsporogenesis in two regions was similar, but pollen abnormality in microspores and pollens of polluted area increased; however, this difference was not significant. We feel that further

investigations are required to prove that environmental pollution was the cause of these changes.

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