

## Original Article

# Identifying the Allergenicity of Maize Pollen in Iran

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## Abstract

**Background:** Maize is a member of the Poaceae family, capable of producing large amounts of pollen grains which may constitute important allergens in spring and summer. The aim of this study was to determine the protein content of maize pollen and its allergenicity in guinea pigs.

**Methods:** The morphology of maize pollens was determined using light microscopy and scanning electron microscopy. The size of separated proteins was obtained by SDS-PAGE. A group of animals were immunized with maize pollen extract and the others were kept as control. After 40 days, the skin prick test was done in animals after blood sampling for counting the eosinophils. The allergenicity of proteins was identified by immunoblotting of transferred bonds using sera from sensitized guinea pigs.

**Results:** Pollen grains showed a spherical, monoporate structure with the scabrate exine surface. The SDS-PAGE indicated a major band of about 50kD. We also showed increase in flare and wheal diameter following skin prick test in sensitized guinea pigs along with an elevated number of eosinophils. The presence of group 13 allergen (Zea m13) with molecular weight of ~ 50kD was found in immunoblotting results.

**Conclusion:** This study showed one protein in maize pollen extract that could be considered as an allergen belonging to group 13 of allergen categories. However, further investigations should be scheduled for precise analysis of the proteins. This allergen can be used for diagnostic or therapeutic purposes (vaccination approaches) in allergic asthma patients.

**Keywords:** Allergenicity, allergen, maize pollen

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## Introduction

Maize is a member of the Poaceae family recognized as the most strategically and economically important plants which are used widely for different purposes such as food industries, extraction of oil and production of starch and ethanol. As one of the major cereal crops with good adaptation to different climate conditions, maize is cultivated in various parts of the world.<sup>1</sup> Allergens are those antigens responsible for clinical allergic diseases. They are usually proteins or glycoproteins capable of inducing synthesis of IgE antibodies. Pollens and their proteins are one of the major prevalent sources of aero-allergens in the world. Despite the existence of more than 1000 different components in grass pollen, only a few can induce IgE-dependent allergic reactions.<sup>2</sup>

Up to now, thirteen groups of grass pollen allergens have been identified. Recent reports indicated that groups one and thirteen are important allergens in maize pollen.<sup>2,3</sup> The greatest reactivity has been shown between group 1 grass pollen allergens and allergen-specific IgE of sera from allergic patients (more than 90%).<sup>2,4,5</sup> Group 13 allergens, with the polygalacturonase structure and molecular weight of 50 to 60 kD, have about 50% reactivity

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with allergic patients' sera.<sup>6,7</sup> Zm1 and Zm13 have been reported as representative maize pollen allergens of group 1 and 13 families in a mature maize pollen.<sup>8,9</sup>

Maize is a monoecious plant (Figure 1). Male and female flowers are produced on a terminal tassel and lateral ears of the plant, respectively. Long branches and the central spike produce rows of short branches or paired spikelets (lower inset), each of the spikelets has two florets having three stamens. Maize is a wind and insect pollinated plant.<sup>10,11</sup>

The *Poaceae* family have been reported as the most important causes of allergy during spring and summer. The major types of allergens belong to the *Panicoideae*, *Chloridoideae* and *Pooideae* subfamilies of the *Poaceae* family.<sup>12</sup>

Good adaptation of maize to different climate conditions has made its cultivation easy and worldwide. Therefore, the aim of this study was to evaluate the maize pollen allergenicity as occupational allergens between farmers exposed to maize pollen.

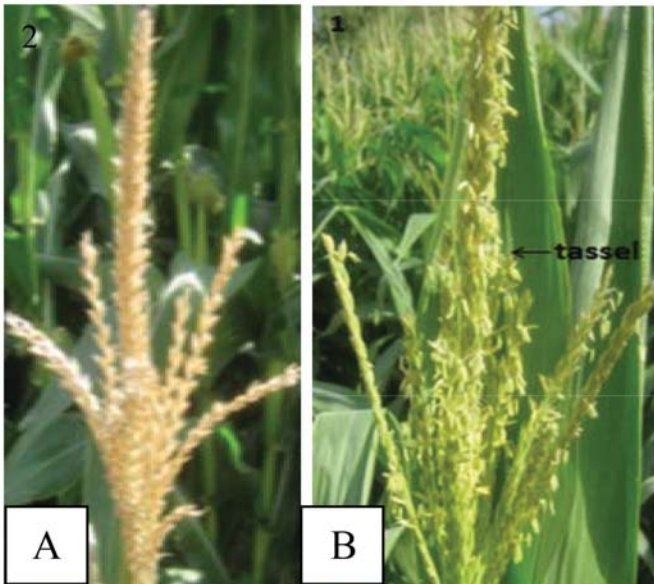
## Materials and Methods

### Scanning Electron Microscopy (SEM)

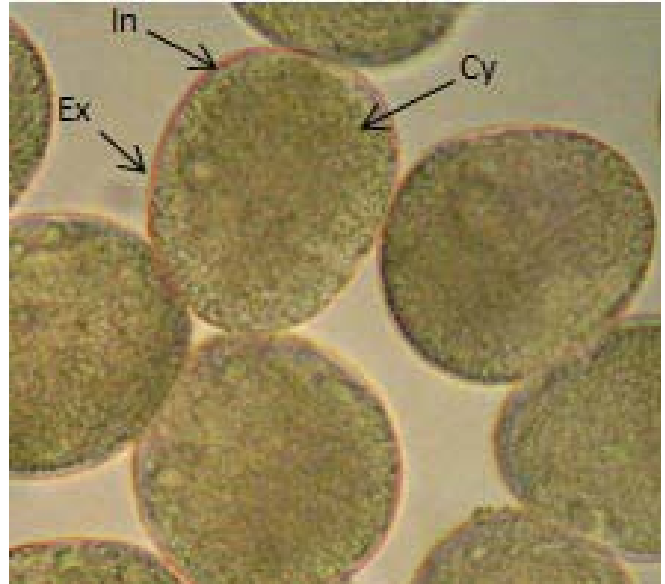
The morphology and structure of pollen were examined by scanning electron microscopy (philipsXL30).

### Preparation of pollen extract

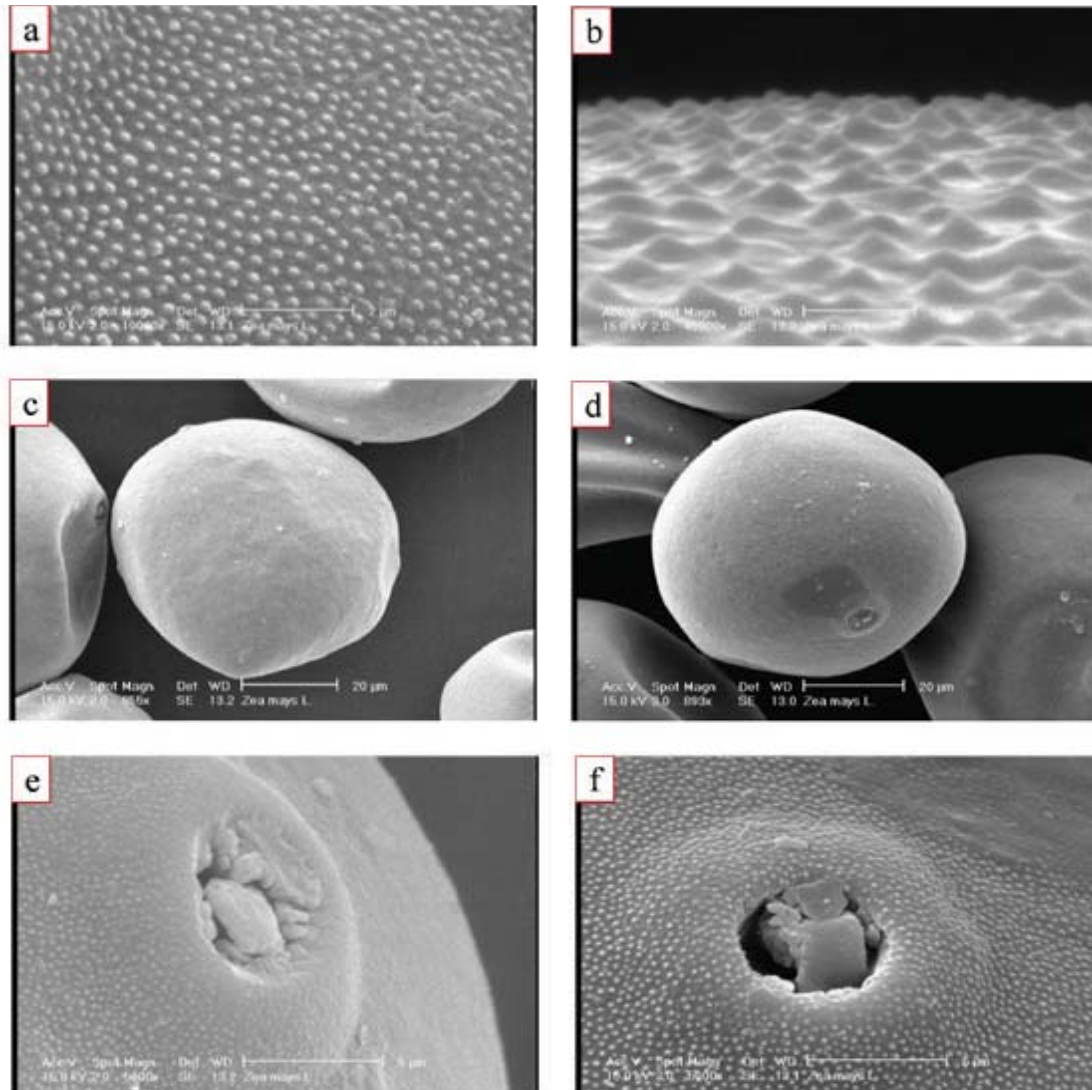
Fresh pollen was collected by shaking the tassels gently over a sheet of paper. Maize pollen (1 g) was incubated overnight in 20 mL of Phosphate Buffer Saline (PBS, PH = 7.2). The soluble fraction was isolated by centrifugation at 11000x for 45 minutes and dialyzed against double-distilled water overnight. The extract was then stored at 4°C until use. The protein content of pollen extract



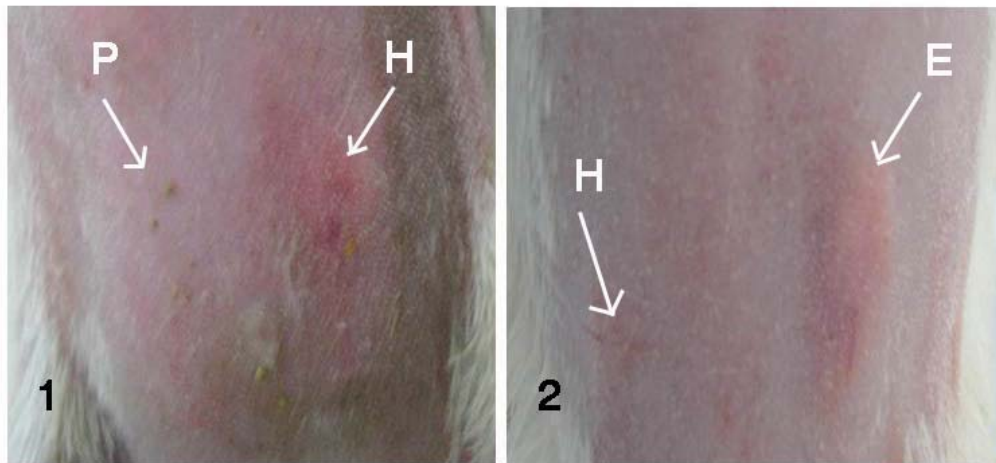
**Figure 1.** General morphology of maize male inflorescence (tassel); **(A)** Opening anthers and exiting pollen grains, **(B)** Anthers has completely opened and pollen grains have exited.



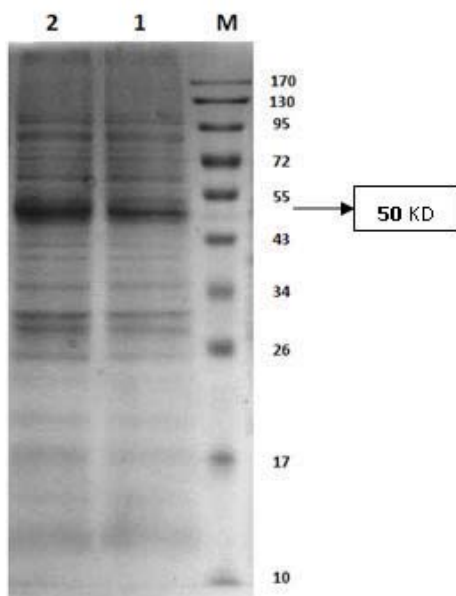
**Figure 2.** Light microscopy image of pollen grains with objective 40x: Ex: exine, Cy: cytoplasm, In: Intine.



**Figure 3.** SEM images of maize pollen grain; **(a) & (b)** spherical pollen with diameter of 63.9  $\mu\text{m}$  on 855x and 893x magnification, respectively, **(c) & (d)** vegetative pore with diameter of 5.34  $\mu\text{m}$  on 5000x magnification, **(e) & (f)** scabrate sculpture of exine surface on 10000x and 40000x magnification, respectively.



**Figure 4.** Wheal and flare in the skin of animals. i.d injection of pollen extract in PBS-immunized group (1) and pollen extract-immunized group (2) of animals caused severe skin reaction in comparison with histamine (H) and PBS (P).



**Figure 5.** SDS-PAGE profile of maize pollen extract with 12.5% gel in different dilutions; (1) 1.25% extract at sample buffer: extract ratio of 40: 60, (2) 2.5% extract at sample buffer: extract ratio of 40: 60.



**Figure 6.** Immunoblotting of extract proteins reacting with IgE in sera from immunized guinea pigs. To show the specific reaction of IgE antibodies with the proteins, the papers were incubated with IgG, polyclonal anti-pig IgE conjugated with horseradish peroxidase. The color was detectable after adding the substrate to the papers.

was determined quantitatively and qualitatively by Bradford protein assay and SDS-PAGE, respectively.

#### Immunization of animals

Twenty-one male guinea pigs of Hartley race weighing 350 g – 400 g and 2 – 3 months of age were divided in 3 groups, each group containing seven animals. Each animal in the first group (the test group) was injected intraperitoneally with 75µL of pollen extract 5% (1g in 20 mL PBS). Animals in the second group (negative-PBS control) were injected with 75 µL PBS in the same place. The third group (negative-untreated control group) was kept without any injection. Injections were performed four times over a 10-day interval.

#### Infiltration of eosinophils and skin prick test

After the last injection, blood was taken by cardiac puncture for

evaluation of absolute eosinophil count. The smear was prepared on slides from blood samples and eosinophils were counted using a microscope. Serum of the remaining blood sample was obtained for immunoblotting and saved at -20°C until use.

The *in vivo* immunological response of pigs was determined using a routine skin prick test. For this part of the study, 20 µL of pollen extract 5% (1g in 20 mL PBS) and 20µL of histamine (1mg in 1mL PBS) were injected intradermally in two separate parts of guinea pigs' abdomen (test/sensitized group). For the control group (control-PBS group), we injected 20 µL of PBS and 20 µL of histamine (1 mg in 1mL PBS) intradermally in two separate parts of guinea pigs' abdomen. We did not administer any injection to the negative-untreated control group. The flare and wheal diameter in the injected sites were evaluated and compared between test and control groups. Reaction to histamine was considered positive in comparison with extract and PBS injections.

**Table 1.** The physical characteristics of maize pollen grain obtained using Scanning Electron Microscopy (SEM), SDS-PAGE and immunoblotting. The percentage of eosinophils in tested and control groups are also indicated.

Parameter	Quantity
Size of diameter of pollen	63.9 $\mu\text{m}$
molecular weight of protein (major band)	$\sim 50\text{kD}$
Allergen group	13
<b>Percentage of eosinophils in groups</b>	
Pollen extract	6.142
PBS	1
Control	1.33

### Serological assessment

Total protein of pollen extraction was measured by Bradford.<sup>13</sup> SDS-PAGE was performed using 12.5% polyacrylamide gels. The resolved proteins were visualized using Coomassie Blue R250 staining, and/or electrophoretically transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Uppsala, Sweden). The membrane was blocked 3 h at room temperature with 5% milk powder in TBS buffer (10 mm Tris-HCl, PH 7.5, 150 mm NaCl and 0.1% Tween 20) and then washed three times for 10 min in TBST (TBS plus 0.1% Tween 20). After washing, the membrane was incubated overnight with 1:7000 diluted sera of test and control guinea pigs followed by washing and incubating for 1 h with 1:15000 dilution of IgG<sub>1</sub> polyclonal anti-pig IgE conjugated with horseradish peroxidase (HRP) (Serotec UK). For developing the protein bands, the membrane was washed again and then treated by the DAB substrate (0.5 mg.ml<sup>-1</sup> diaminobenzidine and 0.1% hydrogen peroxide in TBS).

### Statistical analyses

One-way ANOVA was used to evaluate differences between groups. Tukey HSD was also done to determine which groups differ from the others. All statistical analyses were carried out using SPSS 11.5 (SPSS Inc.). The significance level of 0.05 was considered in this study.

## Results

### Maize and pollen structure

The general morphology of maize male inflorescence (tassel) with the opening anthers and pollen grains is shown in Figure 1. The cytoplasm and inner and outer wall layers (exine) of the pollen grain are indicated on the light microscopy image of pollen grains using a 40x objective lens (Figure 2). The Scanning Electron Microscopy (SEM) images of maize pollen grain showed that the maize pollen has a spherical shape with a diameter of 63.9  $\mu\text{m}$  and is also monoporate with a pore size of 5.34  $\mu\text{m}$  (Figure 3).

### Infiltration of eosinophils and skin tests

The smears of animal blood samples showed a significant increase in the percentage of eosinophils in pollen extract-injected groups compared with control-PBS-injected ( $P = 0.0001$ ) and negative-untreated controls ( $P = 0.005$ ). The mean percentages of eosinophils were  $6.142 \pm 0.81$ ,  $1 \pm 0.44$  and  $1.33 \pm 0.88$  in the pollen extract-injected, control-PBS-injected and negative-untreated controls, respectively. Wheal and flare in the skin of animals immunized with the pollen extract was  $1.0714 \pm 0.1$  in comparison with histamine ( $0.5814 \pm 0.03$ ) and PBS ( $0.5757 \pm$

$0.03191$ ) controls. The allergic reaction to pollen extract injection was significantly higher than that of the PBS-injected ( $P = 0.0001$ ) and untreated control ( $P = 0.00001$ ) groups. The wheal and flare reaction appearing in the skin of animals after injection of extract, histamine and PBS is shown in Figure 4.

### Protein study and serum immunoblotting

The protein content of pollen extract was determined by Bradford and SDS-PAGE. The total protein content was  $1775 \pm 150 \mu\text{g.mL}^{-1}$  (Figure 5). Thus, for injection of 100  $\mu\text{g/mL}$ , we prepared a 5% dilution of pollen extract in PBS.

SDS-PAGE demonstrated the presence of a major band ( $\sim 50\text{kD}$ ) and several minor bands.

Immunoblotting of pollen extract proteins in the presence of sera from immunized guinea pigs revealed that the group 13 allergen in the extract can react with IgE produced in immunized animals (Figure 6).

Table 1 shows the results of tests performed on the maize pollen grain.

## Discussion

Although the potential allergenicity of maize pollen has been investigated in different countries, this is the first evaluation of the influence of climatic conditions on allergenicity of maize pollen in Iran. In our study, the diameter of maize pollens was 63.9  $\mu\text{m}$  in Varamin's maize farms, which was smaller than those reported by Hrabina, et al.<sup>14</sup> The size of pollens can be influenced by different climatic condition in Varamin which affects maize plant to produce small pollens.

Our protein assay and electrophoresis results showed higher amounts of protein and several minor bands in comparison with the report of Wang.<sup>3</sup> This discrepancy between the protein content of our pollens and other studies might be a compensatory condition to tolerate the high difference between day and night temperatures (about 20 °C) in Varamin city which results in production of shock proteins in maize plants. These shock proteins help the plant to cope with stress conditions.<sup>15</sup>

The allergenicity of maize pollen is greatly associated with the protein content of this plant. According to the classification of Arshad, et al. based on the skin test manifestation of allergen extract, we found that the wheal diameter of extract injection in the skin of guinea pigs treated with pollen was three times more than histamine injection which was the intense skin test allergy indicator of pollen extract.<sup>1</sup>

In the present study, guinea pigs sensitized with extract pollen showed clinical symptoms including severe itching, alopecia, decreased sense of olfaction due to inflammation of nasal mucus

as a consequence of allergic rhinitis and increased number of eosinophils in blood. These clinical symptoms are related to allergenicity of maize pollen. Freeman, Gonzalo-Garijo, Mohapatra and Petersen observed that maize pollen-exposed farmers showed allergic symptoms as well as various combinations of oral and ear itching, sneezing, cough and wheezing. There are some studies reported by Mohapatra<sup>6</sup> and the others<sup>16-18</sup> on positive skin test reactions to maize pollen among sensitive patients and also increased IgE reactivity to maize extracted antigens.

Immunoblotting of sensitive guinea pigs' sera showed group 13 allergen. Vollbrecht, et al. reported that different genes are responsible for allergic diversity exists in maize inbred lines, which can create allergenicity potential diversity in maize pollen.<sup>19</sup> Thus, the allergenicity of maize pollen can be associated with two important factors, climate condition and plant genotype which influence the intensity of allergens type.

### Acknowledgment

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