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**Saeed Irian, Ahmad Majd, Aboozar
Hoseinizadeh & Parisa Jonubi**

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A comparative study on the allergenicity, structure, and ultrastructure of two *Acacia* pollen grains in guinea pigs

Saeed Irian · Ahmad Majd · Aboozar Hoseinizadeh · Parisa Jonubi

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Abstract *Acacia saligna* and *Acacia victoria* are native to Australia brought to and grown in Southern Iran. They have since had a widespread distribution and become native in Iran. As people of the region are exposed to the pollen grains from these plants during a 5 months period, this study aimed at investigating the allergenicity of their mature pollen grains. In addition, the structural and ultrastructural as well as the total protein content of the mature pollen grains were analyzed. Pollen grains of *A. saligna* and *A. victoria* were collected from the suburbs of Ahvaz, Iran. Pollen extracts (15 % concentration) were prepared in PBS (pH 7.2), and 4- to 6-week-old male guinea pigs (*Hartly*) were used for allergenicity tests. Skin tests showed a significant increase in flare diameter. Clinical tests also showed a significant change in the levels of eosinophils, neutrophils, and IgE. Comparative analysis of the allergenicity of *A. saligna* and *A. victoria* pollen grains revealed a higher IgE level in the latter. Comparative analysis of pollen characteristics was performed using both light and electron microscopy for sporoderm structure and ultrastructure, respectively. Twelve percent PAGE analysis of the total protein content of mature pollen grains showed a greater number of bands in *A. victoria*, while four bands were common in both species. In

conclusion, this study demonstrates that both *A. saligna* and *A. victoria* pollen grains are allergenic, with a greater allergenicity of *A. victoria* pollen grains. In addition, the structural and ultrastructural as well as the total protein content of the mature pollen grains are revealed, and a potential protein allergen is proposed.

Keywords Pollen grain · Allergenicity · *Acacia* · Ontogeny

1 Introduction

Acacias are among the important tree and shrub species growing in the shores of the Persian Gulf and the sea of Oman. Some species of the genus are natural elements of the region's flora, despite some other species are cultivated by people. Their unique root system allows water absorption from the lower levels of the ground. For this reason, they have been adapted to grow in dry climates. The flowering cycle normally starts early in the spring and continues through the midsummer. Different species of *Acacia* have been used for animal feed, woodworking, preparing flower extracts, pharmacology, and in the tools of farmers and fishers, in particular the boat industry.

Acacia saligna and *Acacia victoria* are native to Australia brought to and grown in Southern Iran. They have since had a widespread distribution and become native in Iran. *A. saligna* is a shrub with woody-

S. Irian (✉) · A. Majd · A. Hoseinizadeh · P. Jonubi
Department of Cell and Molecular Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran
e-mail: irian@tmu.ac.ir

herbaceous branches and colors ranging from green to light brown. Leaves are simple, elongated, and pointy. The inflorescence is capitul type with a light yellow color. The flowering time is mid January to mid April (Milton and Hall 1981). *A. victoria* is a grayish-green tree with woody branches. Leaves are simple and elongated, with white inflorescence. The flowering time is mid March to mid May (Webb et al. 1980). Therefore, the people of this region are exposed to the pollen grains from these plants during a 5 months period.

In the past few decades, the reported cases of the allergic rhinitis and other related diseases like asthma and urticaria have grown at tremendous rate as recorded from various epidemiological studies carried out all over the world (Björkstén et al. 2008). The pollen grains constitute one of the important components of air and are considered as a major causative agent of respiratory disease, as is evident by the spurt of literature from across the world during the last few decades (Clot 2003; Fang et al. 2001).

Allergy to *Acacia* has been reported by several studies. Allergic rhinitis, also known as hay fever, and asthma are allergic reactions commonly observed in wood workers exposed to pollen from *Acacia* or *Acacia* wood (Wood-Baker and Markos 1997; De Zotti and Gubian 1996). Cross-reactivity between *Acacia* tree and Rye grass pollen allergens has also been described (Howlett et al. 1982).

Pollen grains like any other plant cell contain proteins and glycoproteins, a few of which can cause allergy. These allergens normally weight in the range of 10–70 kDa and are localized to different parts of the pollen including ground cytoplasm, intine, exine, and etc. (D'Amato et al. 2005; Knox and Suphioglu 1996). Following their entry into the respiratory system, these allergens are quickly absorbed through the respiratory tract mucus, and immediately result in a Type I allergic reaction. Type I allergic reactions mediated by IgE result in the production of more IgEs as well as eosinophils and neutrophils in the blood. Interaction between the allergen and the IgEs located on the mast cells (in tissues) or the basophils in blood circulation results in the release of inflammatory agents such as histamines, serotonin, heparin, and leukotrienes, which in turn by inducing smooth muscle contraction and increasing vein penetrability result in an inflammation (Wheal) and redness (flare) in the skin, properties known as the allergic reaction index. To

determine the allergy potency of different pollen extracts, normally, skin test, blood eosinophil and neutrophil number, and IgE levels in sensitized and treated animals are measured (Abbas et al. 2007).

The aim of the present study was to further investigate both the structure and ultrastructure of *A. saligna* and *A. victoria* pollen grains, analyze and compare their allergenicity and the total protein content of the mature pollen grains. In addition to gaining more knowledge on the basic science of these plants, such information may assist future investigators in a better use of the plant.

2 Materials and methods

2.1 Allergenicity assays

2.1.1 Sampling

Acacia saligna and *Acacia victoria* (Fabales, Mimosaceae) pollen grains were collected from the suburbs of Ahvaz, Iran. Pollen grains were size-selected using a mesh with holes of 60 µm in diameter.

2.1.2 Animals and sensitization

Male 4 to 6-week-old guinea pigs (*Hartly*), weighting 350–500 g, were purchased from the Pasture Institute of Iran and kept in a room at 25 °C with an 8-h light/8-h dark condition. They were fed a standard laboratory diet and given water with added vitamins. These animals were then divided into two groups of seven animals each and sensitized with 100 µl *A. saligna* and *A. victoria* pollen extracts (containing 50 µg protein in phosphate-buffered saline) with 50 µg Al(OH)₃ added as an adjuvant. Al(OH)₃ was suspended in saline buffer and added to pollen extracts. Treatments were repeated six times, for 3 weeks, twice a week.

2.1.3 Skin test

A. saligna and *A. victoria* pollen extracts were prepared by the method of Sheldon et al. (1967), and skin test was performed on guinea pigs. Each animal was tested with 100 µl of the total extract diluted with 0.05 M phosphate-buffered saline (pH 7.4) containing 50 µg protein without adjuvant. The negative control received 100 µl of buffered saline. Skin reactions were

read 12 h from the beginning of the test and quantified on the basis of flare diameter.

2.1.4 Clinical and serological tests

Prior to each experiment, the guinea pigs were bled once. Blood samples were drawn directly from the hearts. Following treatment with pollen extract, the guinea pigs were anesthetized with chloroform, and blood was then obtained directly from the hearts (Aberg 1989). Sera from all samples were stored at -70°C until used. The sera from seven *A. saligna* and seven *A. victoria* sensitized animals were individually tested for IgE reaction to *A. saligna* and *A. victoria* pollen grains. Sera from seven animals that were not sensitized to these pollen grains were also tested as control sera. Serum total IgE level was determined by ELISA, using an IgE guinea pig ELISA kit (Cusabio Co.) (Mondal et al. 1997). The serum IgE level was expressed in nanograms per milliliter. Blood smears of healthy controls were compared with those of the experimental animals. Total numbers of different kinds of blood cells, specially, eosinophils, and neutrophils were determined in the two groups and statistically analyzed.

2.2 Structural and ultrastructural analysis

2.2.1 Light microscopy

To study pollen wall structures, Acacia pollen grains were subjected to acetolysis analysis using the method of Erdthman (1960) before being examined under a light microscope (Zeiss) (Erdthman 1960).

2.2.2 Scanning electron microscopy

For ultrastructural analysis of *A. saligna* and *A. victoria* pollen grains, purified pollen grains were fixed with glutaraldehyde, postfixed with osmium, dehydrated, and dried prior to gold coating. Coated samples were then analyzed using a scanning electron microscope (XL30, Philips, Holland).

2.3 Protein studies

2.3.1 SDS-PAGE analysis

Extraction of pollen protein from *A. saligna* and *A. victoria* pollen grains was carried out separately at

4°C in Tris-HCl buffer, pH 7.6. A 12 % SDS polyacrylamide gel electrophoresis was performed for the total soluble protein according to the method of Laemmli (1970) (Laemmli 1970). Soluble proteins were extracted in sample buffer (0.125 M Tris-HCl (pH 6.8); 4 % SDS; 20 % glycerol; 10 % β -mercaptoethanol; 0.1 % BPB) with heating for 3–4 min at 100°C before loading. The amount of protein was 10 μg per channel, and the total current applied was 14 mA. The gel was run in Tris-glycine buffer (pH 8.3) with 0.1 % SDS, and calibrated with a marker protein obtained from Sigma. Protein concentration of the pollen extract was determined by the method of Bradford (Bradford 1976).

2.4 Statistical analysis

Comparisons were analyzed using the One-way ANOVA, and significant differences between groups were analyzed by Tukey's multiple-range test. P value <0.05 was considered significant.

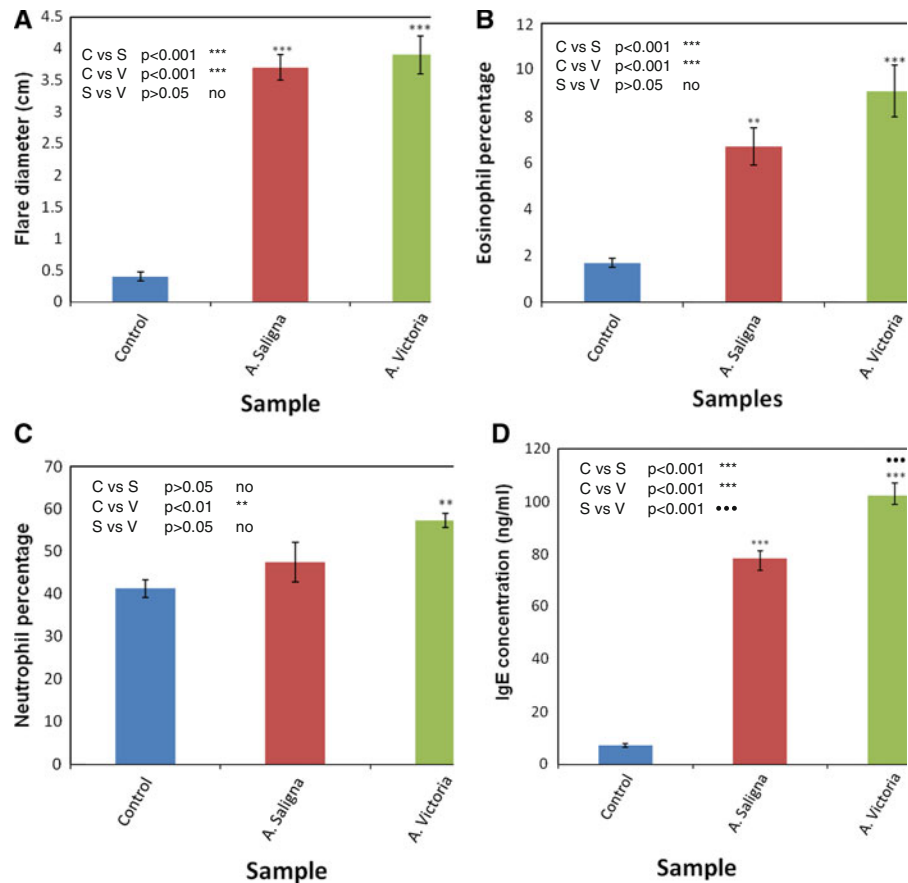
3 Results

3.1 Allergenicity

The maximum sensitivity to the allergenic mature pollen grains included a flare with a 3.9 and 3.7 cm in diameter for *A. victoria* and *A. saligna*, respectively. Despite a lack of significant difference between the two pollen grain extracts, these values were significantly ($P < 0.0001$ and $P < 0.001$, respectively) different than that of the control animals (Fig. 1a).

Counts of eosinophils showed a significant ($P < 0.001$ and $P < 0.01$ for *A. victoria* and *A. saligna*, respectively) difference with that of the control line (Fig. 1a), while those of neutrophils were only significantly higher in *A. victoria* ($P < 0.01$) and not in *A. saligna* (Fig. 1b–d). Compared to the control, IgE levels were significantly higher in both *A. victoria* ($P < 0.001$) and *A. saligna* ($P < 0.001$) extract-treated sera. Comparative analysis between *A. victoria* and *A. saligna* extract-treated sera showed no significant difference in the counts of eosinophils and neutrophils; however, IgE levels were significantly ($P < 0.001$) higher in *A. victoria* than in *A. saligna* pollen grains (Fig. 1d).

Fig. 1 **a** Pollen extract skin test showing flare diameter. **b** Serological data for the percentage of eosinophils. **c** Serological data for the percentage of neutrophils. **d** Serological data for blood IgE levels. Samples: Control: animals injected with PBS buffer; *A. saligna*: animals injected with *A. saligna* pollen extract in PBS buffer; *A. Victoria*: animals injected with *A. victoria* pollen extract in PBS buffer. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$



3.2 Structural and ultrastructural analysis

Acetolysis analysis revealed *A. saligna* and *A. victoria* pollen grains in the form of a collection of interconnected grains called polyad. The polyad structure was both spheroidal and heteropolar in shape with a diameter of 45–50 μm , while each pollen grain had a diameter of 7 μm . SEM images revealed *A. victoria* and *A. saligna* pollen grains with a pseudocolpexine which was deeper and more pronounced in the latter. The exine surface was of a *Psilate-Foveolate* type in both pollen types which was again more pronounced in the later (Fig. 2a–g).

3.3 Protein studies

Bradford assay performed on total protein, extracted from *A. saligna* and *A. victoria* pollen grains, indicated a 0.31 and 0.74 mg/g protein concentrations, respectively. Twelve percent SDS-PAGE analysis revealed several bands in the range of 10–100 kDa. There were more protein bands in *A. victoria* than *A. saligna*. Two bands of

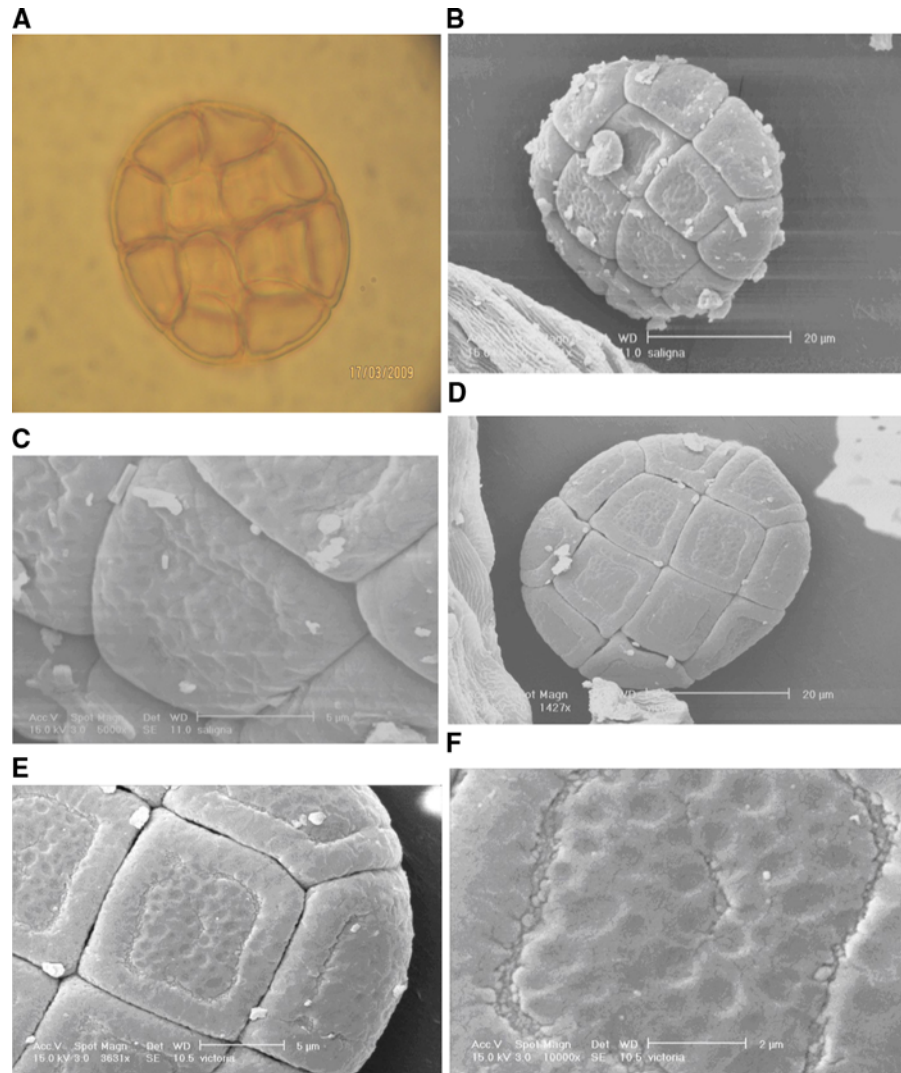
18, 40 kDa were only seen in *A. victoria*, while two bands of 23 and 639 kDa were *A. saligna* specific (Fig. 3).

4 Discussion

4.1 Allergenicity

In the present study, based on the results of our skin and clinical tests performed on guinea pigs for the allergenicity of pollen grains, it appears that *A. saligna* and *A. victoria* pollen grains are allergenic. These included a strong reaction following the skin test; a rise in blood eosinophil levels; the presence of eosinophils in eye and nasal secretions (Rothenberg 2007); and a rise in blood IgE levels (Majd et al. 2004; Onishi et al. 2007), all of which are indicative of the allergenicity of *A. saligna* and *A. victoria* pollen grains. Comparing the allergenicity of *A. saligna* and *A. victoria* pollen grains, it appears that *A. victoria* pollen grains are more allergenic than those of

Fig. 2 **a** Light microscopy image of the acetolyzed *A. saligna* pollen grain (mag. $\times 100$). **b** SEM showing *A. saligna* pollen grains. **c** SEM showing decorations on the exine layer of *A. saligna* pollen grain. **d** SEM showing *A. victoria* pollen grains. **e**, **f** SEM showing decorations on the exine layer of *A. Victoria* pollen grain



A. saligna, due to a higher IgE level in the former, which means that a significant amount of protein allergens is produced in *A. victoria* pollen grains.

Several recent studies have shown that Acacia pollen grains constitute some of the major sensitizing aeroallergens in the Persian Gulf. Date palm pollen (*Phoenix dactylifera*) has been shown to cross-react with antigens from Sydney golden wattle (*Acacia longifolia*) pollen (Kwaasi et al. 2002). Of the 306 allergic rhinitis patients who were evaluated for their skin prick test response to 27 common regional aeroallergens in Iran, *Acacia longifolia* pollen was positive in 29.3 % (Fereidouni et al. 2009). In a study of 327 adult patients, with diseases of suspected allergic origin, who were examined in a hospital in the

United Arab Emirates, skin prick testing showed that among the 244 patients (74.6 %) with positive results, Acacia was positive in 25.6 % (Bener et al. 2002). In a study of aeroallergens in sandstorm dust investigated in Riyadh, Saudi Arabia, Acacia was among the most abundant aeroallergens (Kwaasi et al. 1998). In a different study in Saudi Arabia, of 1,159 patients tested for sensitization to inhalants, Acacia was positive in 29 % (Suliaman et al. 1997).

4.2 Structural and ultrastructural analysis

Structural and ultrastructural analyses of *A. saligna* and *A. victoria* pollen grains using both light and scanning electron microscopy revealed the existence

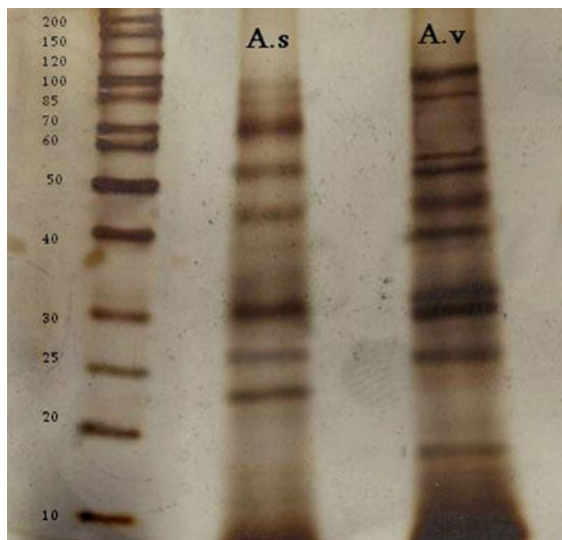


Fig. 3 Image of the stained SDS-PAGE gel showing bands of the total protein extracted from *A. saligna* and *A. victoria* pollen grains (A.s and A.v) and the molecular marker (left) (sizes are in kDa)

on the exine layer of a pattern of striation, and decorations. In general, each pollen grain in a polyad had an uneven surface with a tetragonal pseudocolpi at its center, wherein both antigenic and allergenic materials could accumulate (Heslop-Harrison 1966). The existence of such decorations on pollen grains of the family Mimosaceae and the *Acacia* genus has also been reported by others (Caccavari 2002; Tantawy et al. 2005).

4.3 Protein analysis

Electrophoretic (SDS-PAGE) analysis of *A. saligna* and *A. victoria* total pollen protein content revealed several different bands in the range of 10–100 kDa, while the number of different bands in the latter was greater. Howlett et al. (1982) reported on the cross-reactivity between *Acacia* species and *Lolium perenne* (ryegrass) pollen grains (Howlett et al. 1982), which might be due to the presence of similar proteins in different pollen extracts. Ryegrass serves as an important source of seasonal allergens in hot and temperate climates. This plant contains 17 allergens with MWs ranging from 11 to 86 kDa with a strong reactivity towards IgE. Allergens LoLp1 with a MW ranging from 28 to 35 kDa (Singh et al. 1990) and LoLp5 with a MW of 31 kDa are among its major

allergens (Singh et al. 1991). Since *A. victoria* pollen grains showed a stronger allergenicity than those of *A. saligna*, and the band with a higher density in the former compared to the latter corresponded to the 27 kDa protein, it appears likely that the 27-kDa polypeptide, detected in the present study, represents a strong candidate as one of the several factors responsible for the allergenicity of *A. victoria* and *A. saligna* pollen grains, a finding that is in line with our previous report on *A. farnesiana* pollen grains (Irian et al. 2012). These predictions may further be tested through both immunoblot and clinical analyses.

5 Conclusion

The present investigation used different tests, performed on guinea pigs, to demonstrate the allergenicity of *A. saligna* and *A. victoria* pollen grains. Comparative analysis of pollen allergenicity showed a greater allergenicity of *A. victoria* pollen grains than those of *A. saligna*. In addition, the structural and ultrastructural as well as the total protein content of the mature pollen grains were revealed, and a potential protein allergen was proposed.

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