

Immunocytochemical localization of allergenic proteins from mature to activated *Zygophyllum fabago* L. (Zygophyllaceae) pollen grains

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Received June 27, 2001

Received in revised version October 10, 2001

Accepted November 3, 2001

Zygophyllum – pollen grain – allergenic proteins –
cytochemical immunolabelling – electron microscopy

***Zygophyllum fabago* L. (Zygophyllaceae) can be found in the Middle East, in North Africa and in the arid zones of the Mediterranean region. It easily establishes itself in new regions, and is considered an invasive plant. They undergo ambophilous pollination, as there is a relationship between this type of pollination and its allergenic incidence. A combination of transmission electron microscopy with immunocytochemical methods was used to localize allergenic proteins during hydration and activation processes. Germination was induced in vitro for 1, 2, 4, 6, and 30 min. The activated proteins reacting with antibodies present in human sera from allergenic patients are found in the cytoplasm, intine, exine and exudates from the pollen grains. The activation time plays an important role on the labelling intensity. Labelling of allergenic proteins was abundant at 1 and 2 min of activation, and decreased at 4 and 6 min. The rapid activation and release of the allergenic proteins appears to be the main cause of allergenic activity of *Z. fabago* pollen grains.**

Abbreviations. BSA Bovine serum albumin. – IgE Immunoglobulin E. – RER Rough endoplasmic reticulum. – SEM Scanning electron microscopy. – TBS Tris-buffered saline. – TEM Transmission electron microscopy. – UA-LC Uranyl acetate and lead citrate.

Introduction

It is known that people are in contact with approximately 10 µg pollen grains per year that are transported in the air. While this may seem like a small quantity, it is enough to cause pollinosis in 20% of the population in developed countries (Burney, 1993). In fact, during the past 10 years, pollinosis has doubled in the European Union, and important efforts have been carried out to develop new techniques to mitigate its consequences (D'Amato et al., 1998). Inhalation of pollen grains leads to allergenic rhinitis and type 1 asthma via IgE. The etiology of this phenomenon is due to the presence of substances that can induce anaphylactic processes in susceptible subjects. Free amino acids, proteins and enzymes contained in pollen grains are found in the cytoplasm and in the inner (intine) and outer (exine) wall of the pollen grain (Knox, 1971, 1973; Knox and Heslop-Harrison, 1969, 1970, 1971a, b, 1976). They are essential for pollen-stigma recognition, formation of the pollen tube, and its passage through the style in order for fertilization to take place (Knox, 1984). A considerable number of low-molecular-weight glycoproteins have been located by immunocytochemical techniques in the cytoplasm and in the exine of the mature pollen grains of *Poaceae* (Knox et al., 1970; Knox and Heslop-Harrison, 1971a, b; Howlett and Clarke, 1981; Vithanage et al., 1980, 1982; Abadie et al., 1988; Staff et al., 1990; Márquez et al., 1992, 1997a, b), *Cupressaceae* (Southworth et al., 1988; Miki-Hirosige et al., 1994), *Betulaceae* (Grote, 1991; Grote et al., 1993, 1999; El Ghazaly et al., 1999), and *Urticaceae* (Casas et al., 1996). In the preceding stage of germination, during the activation of the pollen grains, these proteins have been detected in the intine and in the exudates of *Poaceae* (Márquez et al., 1992, 1997a, b), *Betulaceae* (Grote et al., 1993, 1999; El-Ghazaly et al., 1999) and *Urticaceae* (Casas et al., 1996).

Zygophyllum fabago L. (Zygophyllaceae) can be found in the Middle East, in North Africa and in the arid zones of the

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Mediterranean region (Tutin et al., 1968). *Z. fabago* is easily established, and it is considered an invasive plant. It is currently considered a potential hazard on the Coast of California (USDA, 2001). It grows to a small wood bush with numerous branches that measures 60–80 cm in height, with a flowering period that lasts from May to August. *Z. fabago* is predominantly considered an insect pollinated plant. Nevertheless the intermediate, ambophilous pollination, would better characterize *Z. fabago* (Castells et al., 1999; Castells, 2000). The presence of the pollen in the atmosphere may also be related to the exertive disposition of the stamen and the abundant biomass of this plant (Belchí et al., 1997; Castells, 2000). An aerobiologic study in Cartagena, Spain, found that *Z. fabago* pollen grains accounted for 6% of the total airborne pollen during the period of pollination, and about 15% of patients who suffer from a clinic allergy were positive to *Z. fabago* in this area (Belchí et al., 1997, 1998). Sensitisation to *Z. fabago* was detected in a high percentage of patients with a positive cutaneous response. Serum specific IgE levels and provocation tests for this species were also positive, although it is not a predominant aerobiological taxon (Belchí et al., 1997, 1998).

The aim of this study was the immunocytochemical localization of the allergenic proteins found in the *Z. fabago* pollen grains during the hydration and activation processes.

Material and methods

Plant material

Mature anthers of *Z. fabago* collected from the area surroundings Cartagena (Spain) in the spring of 1999 were used.

SEM

Pollen grains were acetolysed. The pollen grains were sputtered with a gold coat (Sputter Coater Polaron E 5000), and observed with a HITACHI S 2300 scanning electron microscope. For the palynological terminology we refer to Punt et al. (1994).

TEM

Activation. Hydration and activation of mature pollen grains was induced in vitro by immersion in a 0.7% agar germination medium (Walden, 1993), for 1, 2, 4, and 6 min at room temperature to supply a humidity and temperature conditions similar to receptive stigma. After each specified time the process was interrupted and anthers were removed from the germination medium and fixed.

Fixation and embedding. LR White embedding: mature anthers and pollen grains activated for 1, 2, 4, and 6 min were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde solution in 0.1 M cacodylate buffer for 2 hours at room temperature. They were next washed in 0.1 M cacodylate buffer for 30 min. This was followed by dehydration in an alcohol series and embedding in LR White resin. Spurr embedding: mature anthers were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer for 2 hours at room temperature. They were then washed in 0.1 M cacodylate buffer for 30 min and postfixed with 1% osmium tetroxide in 0.8% phosphate-buffered $K_3Fe(CN)_6$ for 3 hours at 4°C. This was followed by dehydration in an alcohol series and embedding in Spurr's resin. Sections of the blocks containing the samples were obtained with a Reichert-Jung Ultracut E. These sections were mounted on gold grids.

Human sera. Sera from allergic patients with specific IgE and serum from a non-allergic subject with negative specific IgE were supplied by the University Hospital in Murcia (Spain). Pollen extract: The purity of collected pollen was 99% estimated by optic microscopy. Pollen extracts were prepared with the standard weight/volume method. The protein percentage of the pollen extract was determined by the Bradford method (Belchí et al., 1997, 1998). Serum specific IgE assay was

performed: Solid-phase antigen was obtained by coupling the extract solution (10 mg/ml) to 6 mm diameter CNBr-activated paper disks, according to Ceska and Lundqvist (1972). RAST was performed by the method of Wide et al. (1967) with Phadezym Trazer reagents (Kabi Pharmacia Diagnostics AB). The nitrocellulose blots of the pollen protein revealed five IgE-binding protein bands of approximate molecular masses of 60, 41, 38, and 15.5/14.7 kDa, respectively (Belchí et al., 1998). Two sera with specific IgE equivalent levels (>17.5 PRU/ml) were used (Belchí et al., 1998). Experiments were performed with two similar sera to obtain replicates of the values. Serum of a nonatopic patient was used as control (Belchí et al., 1998).

Immunogold labelling. For detection of allergens (IgE), ultrathin sections were incubated at room temperature on drops of 5% BSA in 0.1 M TBS for 15 min, followed by sensitised patient sera for 2 h. After three 10-min washes, sections were incubated for 1 h with a second antibody bridge, anti-human IgE developed in a goat (Sigma), diluted 1:40 in TBS. After three 10-min washes with drops of 0.1 M TBS, sections were incubated for 1 h with the antibody conjugated to 10 nm diameter colloidal gold particles, using anti-IgG (goat) developed in a rabbit (Bio Cell), using a 1:60 dilution in TBS. This was followed by three washes with drops of 0.1 M TBS for 5 min, two washes with distilled water, and air-drying. For the control of specific patient antibody, the patient sera were replaced by non-allergic subject serum. In the control for non-specific binding of the antibody conjugated to colloidal gold, the patient sera and the anti-human IgE were omitted. For the control of non-specific binding of the bridge antibody, the patient sera were omitted. Finally, the sections were stained with uranyl acetate for 15 min and lead citrate for 5 min and observed in a Philips EM301 transmission electron microscope.

Results

Similar labelling was obtained from both samples with two equivalent sensitised patient sera. Gold particles are not detected in the controls (see Fig. 4c).

Mature and dehiscent pollen grain

Pollen grains are isopolar, suboblate, small ($14-16 \mu\text{m} \times 16-20 \mu\text{m}$) and 3-zonocolporate. Ectocolpi with sharp ends limit a reduced polar area and with the margins and colpus membrane smooth, the endopore is longolate. The exine is reticulate with lumina narrower than muri (Fig. 1b). The sporoderm is formed by a columellar ectexine, with incomplete tectum, well-defined foot layer, thin endexine and fibrillar intine (Figs. 1a, 4a, b and (Castells et al., 1999)). The cytoplasm of the vegetative cell contains numerous, spherical and peripheral mitochondria. However, mitochondria are not found in the endoapertural zones. Spherical plastids are located in the centre of the cell, and cisterns of the RER are arranged in stacks associated with lipid globules, that are close to the vegetative nuclei. The generative cell is simple, small and fuse-shaped (Fig. 1a).

Scarce and no significant labelling is observed in the pollen walls, exine and intine (Figs. 1c, 3a). In the cytoplasm, grouped and well-defined immunolabelling is observed in the basolateral membranes of the RER and near to the stacks of the RER (Fig. 5a).

1 minute activated pollen grain

Abundant allergenic proteins are observed suddenly and extensively in the hydrated and swelled intine at the endoporus level (Fig. 1d). These proteins are released out of the pollen grain in the exudates (Fig. 1d). In the ectocolpus, the endexine is significantly labelled (Fig. 3b), whereas the intine is labelled abundantly (Fig. 3b). The mesocolpial intine shows an exten-

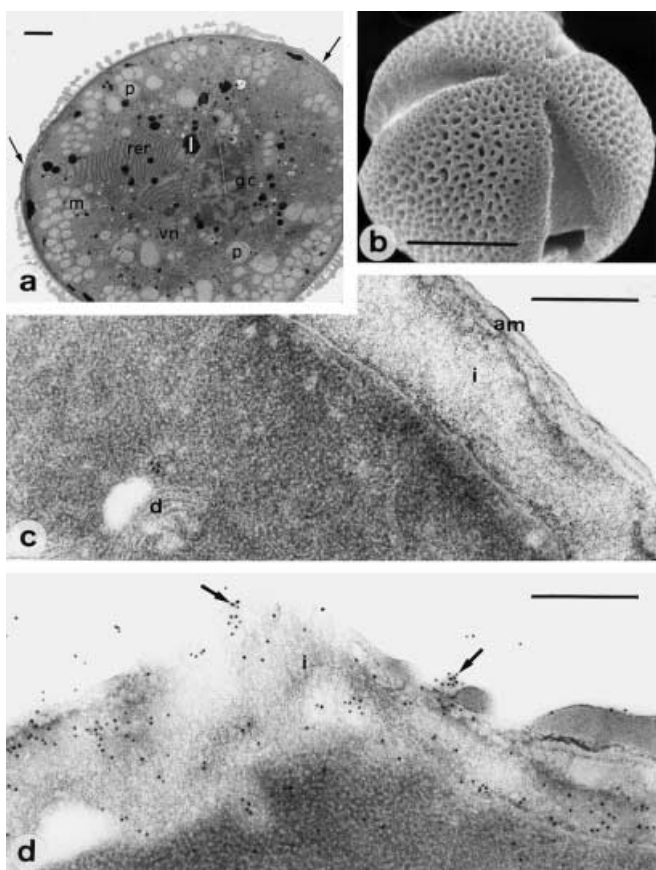


Fig. 1. Morphology of the *Z. fabago* pollen grain (SEM and TEM). **a.** TEM. Section of entire mature pollen grain. Vegetative cell with numerous, spherical and peripheral mitochondria (m), locations without the endoapertural zones (arrows); spherical plastids (p) in the centre of the cell; cisterns of the rough endoplasmic reticulum arranged in stacks (rer) associated with lipid globules (l) located close to the vegetative nucleus (vn) and to the simple, small and fuse-shaped generative cell (gc). **b.** SEM. General equatorial view of acetolysed pollen grain. **c.** TEM. Endoporus. Mature pollen. Detection of allergens by incubation with serum 1. Scarce labelling in the intine (i), apertural membrane (am), dictyosome (d) and peripheral cytoplasm is observed. **d.** TEM. Endoporus. Pollen activated for 1 minute. Detection of allergens by incubation with serum 1. Extensive colloidal gold particles associated with intine (i) and exudates (arrows). Stain: UA-LC. Spurr embedding (a), LR White embedding (c, d). Bars 3 μ m (a), 5 μ m (b), 0.25 μ m (c, d).

sive labelling, the ectexine and the glycocalix show a intense labelling, but it is scarce in the endexine (Fig. 4a). Material extruded from the pollen grains is found extensively labelled, as well in the apertural and the mesocolpial regions (Figs. 1d, 3b, 4a).

Extensive labelling is observed in the vegetative cytoplasm in the nucleus, in the RER, in the cytoplasm vesicles, in the dictyosomes, in the dictyosome vesicles and forming aggregates. In the generative cell, the allergenic epitopes are located in the wall, the nucleus and vesicles (Fig. 5b).

2 minutes activated pollen grain

In a cross section, allergens are extensively located in the endoapertural intine and in the pollen exudates, similar as in pollen grain activated for 1 minute (Fig. 2a). Abundant aller-

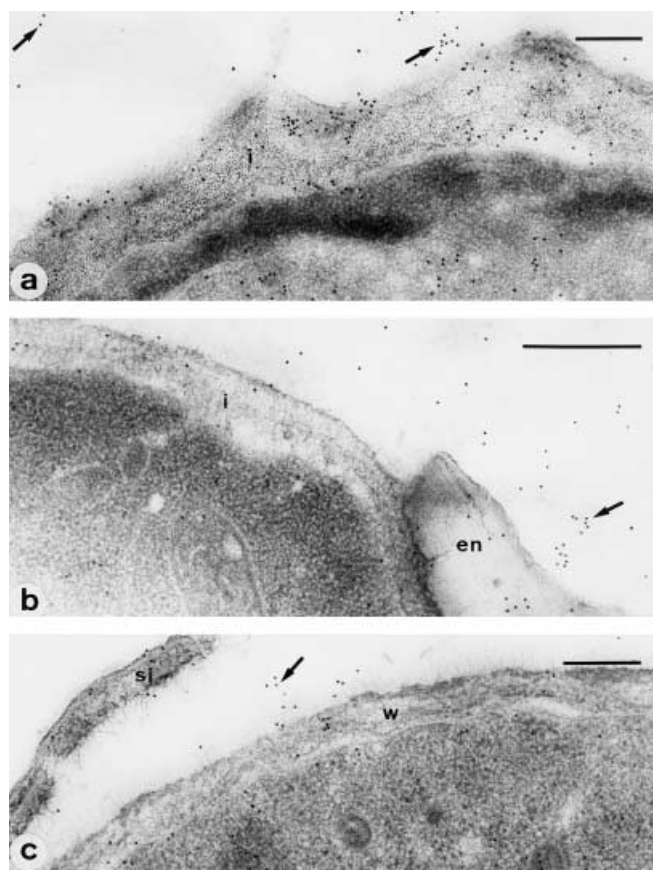


Fig. 2. Endoporus. Pollen activated for 2 minutes. Detection of allergens by incubation with serum 1. Extensive colloidal gold particles in the intine (i) and in the exudates (arrows). **b.** Endoporus. Pollen activated for 4 minutes. Detection of allergens by incubation with serum 2. Intense gold labelling associated with the hydrated intine (i); colloidal gold particles located at the apertural side of the endexine (en). Abundant labelling in the exudates (arrow). **c.** Endoporus. Pollen activated for 6 minutes. Detection of allergens by incubation with serum 2. Intense labelling in the separated intine (si) and slight labelling in the pollen tube wall (w) and pollen exudates (arrow). Stain: UA-LC. LR White embedding. Bars 0.25 μ m (a), 0.5 μ m (b, c).

genic proteins are located in the ectoapertural region (endexine and intine) as well as in the material exuded by the pollen grain (Fig. 3c). In the mesocolpium, the exine and intine are labelled abundantly. Intense labelling is detected in the endexine and extensive labelling is shown in the expelled material (Fig. 4b).

The cytoplasm is significantly labelled, apparently at random, but constantly. Labelling is also found in the basolateral membrane of the cisterns of the RER (as well as inside of them, and frequently in some of their ends), in the cytoplasmic vesicles, dictyosomal vesicles, and in the generative cell wall (Fig. 5c).

4 minutes activated pollen grain

In the endoporus region, intense gold labelling is associated with the hydrated intine. In the mesocolpium slight labelling is detected. Labelling of the exudates appears slightly decreased, but it is abundant yet (Fig. 2b), and colloidal gold particles are located at the apertural side of the endexine (Fig. 2b).

Colloidal gold particles are still located in the cytoplasm, especially in the stacks of the dilated cisterns of the RER, both

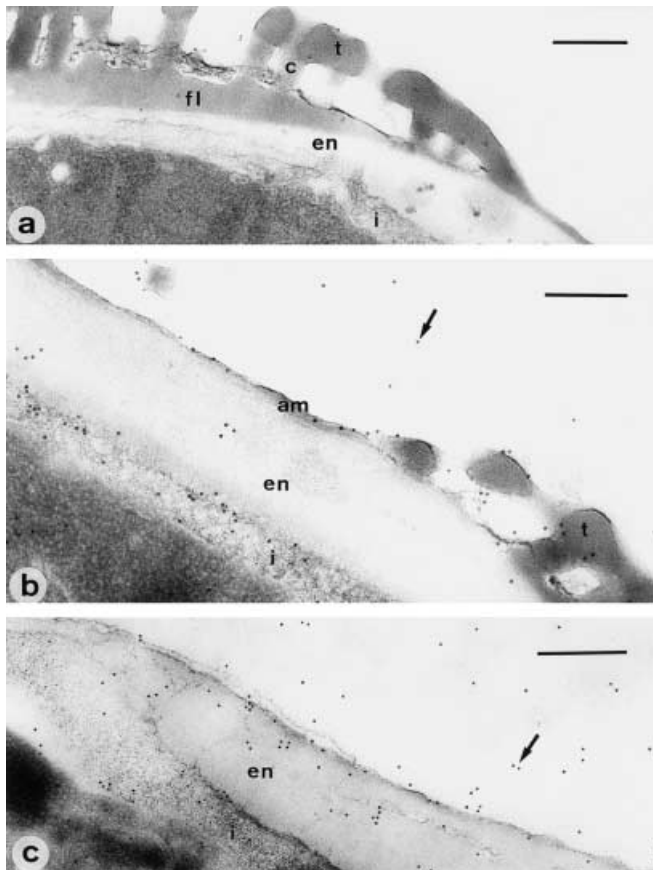


Fig. 3. Ectocolpus. Detection of allergens by incubation with serum 1. **a.** Mature pollen. Scarce labelling in the tectum (t), columellae (c), foot layer (fl), endexine (en), intine (i) and cytoplasm. **b.** Pollen activated for 1 minute. Abundant gold labelling in the tectum (t), apertural membrane (am), and intine (i). Intense labelling in the endexine (en) and extensive labelling in the pollen water-soluble exudates (arrow). **c.** Pollen activated for 2 minutes. Abundant colloidal gold particles in the endexine (en), in the exudates (arrow), and in the intine (i). Stain: UA-LC. LR White embedding. Bars 0.5 µm (a), 0.25 µm (b, c).

in the lumen and in the membranes, and in the dictyosomes and their associated vesicles and other cytoplasm bodies, but the labelling is less abundant than in the previous states (Fig. 5d).

6 minutes activated pollen grain

The presence of allergens decreases notably in the pollen grain when activated for 6 minutes with the initial pollen tube protruding. Intense immunolabelling is detected in the separate intine at the germinal pores, and slightly in the pollen tube wall. Allergenic proteins, which have been released from the pollen grain, are still less located in the pollen exudates (Fig. 2c).

The cytoplasm is still significantly immunolabelled. Colloidal gold particles are randomly arranged in the cytoplasm (Fig. 5e).

Qualitative evaluation of immunogold labelling intensities

A qualitative evaluation of immunogold labelling intensities of the allergenic proteins from the mature pollen grain to the pollen grain activated for 6 minutes is given in Table I.

There are scarce gold probes in the intine in the mature pollen grain. The highest concentration of colloidal gold particles in the endoapertural intine is observed in the pollen

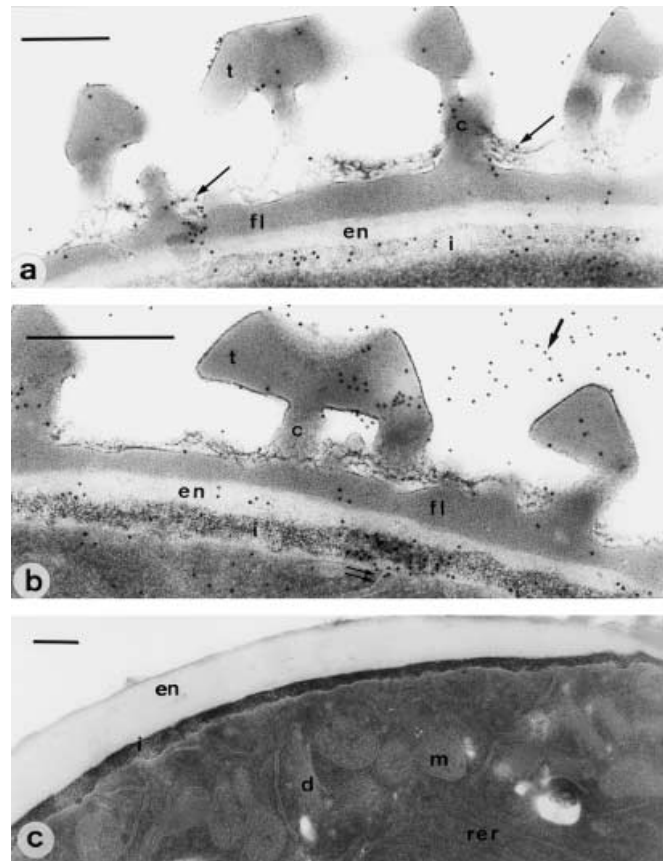
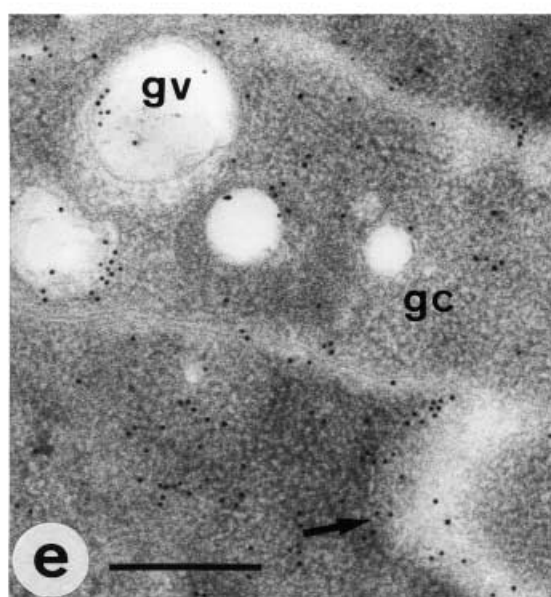
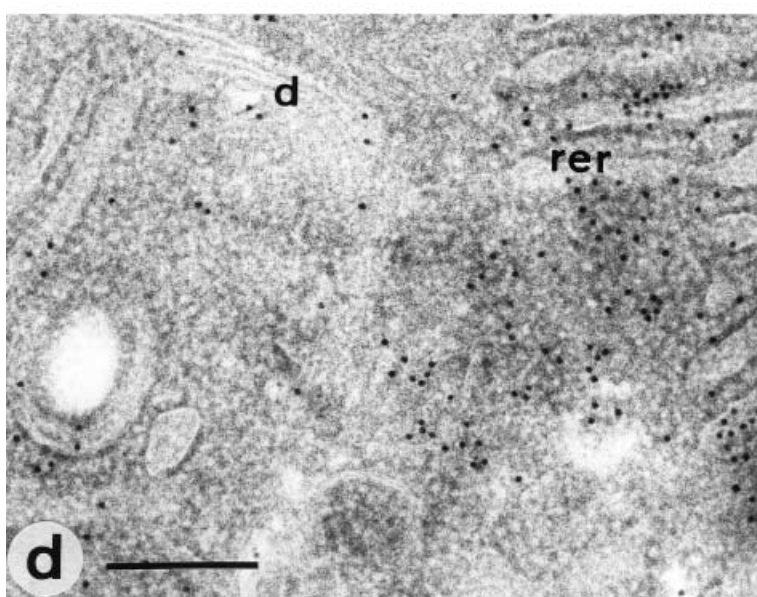
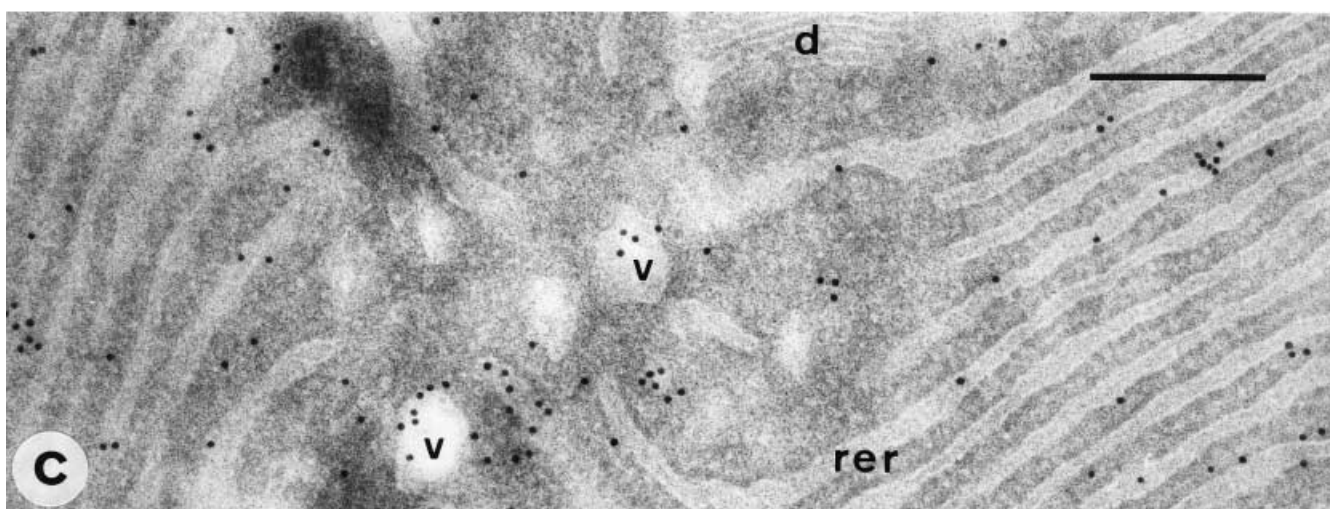
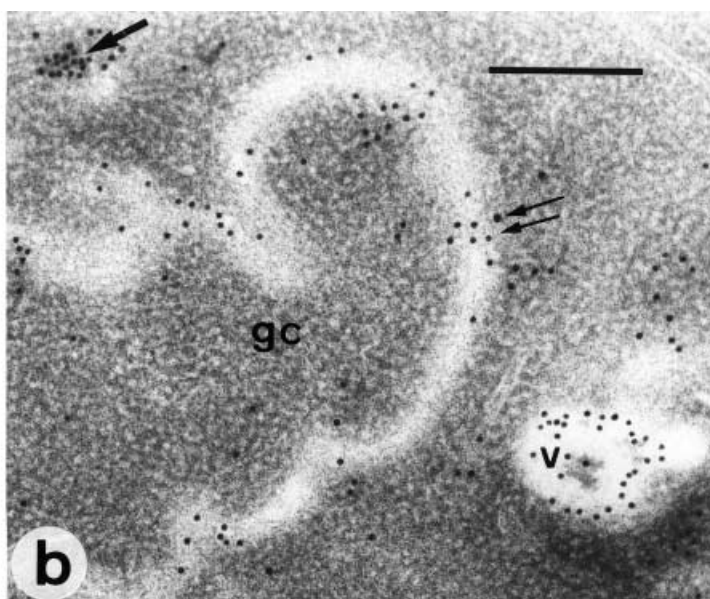
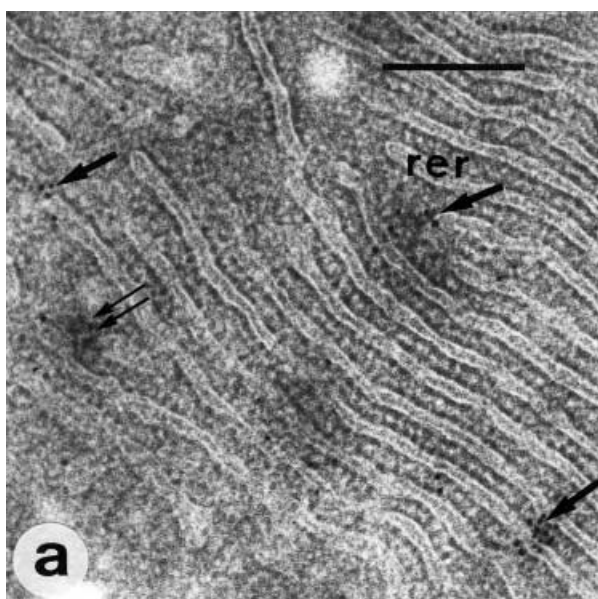


Fig. 4. Mesocolpium. Detection of allergens by incubation with serum 1. **a.** Pollen activated for 1 minute. Note the sporoderm labelling: extensive in the intine (i), intense around the tectum (t), in the columellae (c), foot layer (fl) and glycocalyx (arrows), and scarce in the endexine (en). **b.** Pollen activated for 2 minutes. Abundant labelling in the tectum (t), and in the intine (i); intense labelling in the endexine (en), columellae (c) and foot layer (fl). Exudates show an extensive labelling (arrow). Note gold particles in the cistern of the RER in close contact with plasmalemma (double arrow). **c.** Ectocolpus. Control experiment of pollen activated for 1 minute. Pollen wall and cytoplasm after incubation leaving out the patient serum. Both pollen compartments are free of gold particles. Endexine (en), intine (i), mitochondria (m), dictyosome (d), RER (rer). Stain: UA-LC. LR White embedding. Bars 0.25 µm (a, c), 0.5 µm (b, c).

Fig. 5. Cytoplasm. Detection of allergens by incubation with serum 1 (b–e) and serum 2 (a). **a.** Mature pollen. Scarce, grouped and well-defined immunolabelling in the basolateral membranes (arrows) of the RER (rer), and near to the stacks of the RER (double arrows). **b.** Pollen activated for 1 minute. Extensive immunolabelling mainly associated with the vegetative cytoplasmic vesicles (v), generative cell (gc) and generative cell wall (double arrows). Abundant aggregates of colloidal gold particles in the vegetative cytoplasm (arrow). **c.** Pollen activated for 2 minutes. Extensive labelling in the stacks of the RER (rer) and cytoplasmic vesicles (v), and less in the dictyosome (d). **d.** Pollen activated for 4 minutes. Abundant colloidal gold particles associated with the released vesicles from the forming face of the dictyosome and dictyosome membranes (d), and dilated cisterns of the RER (rer). **e.** Pollen activated for 6 minutes. Abundant immunolabelling in the generative cell vesicles (gv) and in the generative cell (gc) wall (arrow). Note the absence of colloidal gold aggregates. Stain: UA-LC. LR White embedding. Bars 0.25 µm (a–d), 0.5 µm (e).



Tab. I. Qualitative evaluation of immunogold labelling intensities of mature and activated *Zygophyllum fabago* pollen grains.

	Mature	1 min	2 min	4 min	6 min
Intine					
Endoporus	±	++++	++++	++	++
Ectocolpus	—	+++	+++	+	±
Mesocolpium	—	++++	+++	±	±
Endexine					
Apertural endexine	±	++	+++	+++	++
Mesocolpial endexine	—	±	+	—	—
Mesocolpial ectexine (tectum, columellae, foot layer)	—	+++	++++	+	+
Pollen grain exudates	—	++++	++++	+++	+
RER	±	++++	++++	+++	+
Cytoplasmic vesicles	—	++++	++++	+++	+++
Cytoplasmic aggregates	±	+++	+++	+	—
Generative cell	—	++++	++++	+++	+++

Labeling intensities: — no labelling, ± scarce, + slight, ++ intense, +++ abundant, ++++ extensive.

grain activated for 1 and 2 minutes, and it decreases at 4 and 6 minutes. In the mesocolpial intine, the labelling is higher when pollen grain activation is 1 and 2 minutes than 4 and 6 minutes (Table I). The immunolabelling of the apertural endexine increases extensively when the pollen grain is activated for 1, 2 and 4 minutes and decreases significantly at 6 minutes after the beginning of the hydration. Slighted immunolabelling is observed in the mesocolpial endexine when activated for 2 minutes (Table I). The pollen grain ectexine activated for 2 minutes shows the highest concentration of colloidal gold particles. Gold probes in the pollen grain ectexine activated for 1 minute compared with pollen grain activated for 4 and 6 minutes are substantially increased (Table I). There is a sudden and intense labelling of exudates corresponding at 1 and 2 minutes of the pollen grain activation, and it only slightly decreases when pollen grain activation time is 4 minutes (Table I).

Generally, the cytoplasm shows scattered and uniform labelling, although the labelling intensity can be compared for the different compartments and organelles of the vegetative cell as well as on the generative cell. The labelling in the RER, in the cytoplasm vesicles, in the generative cell as well as the presence of the colloidal gold particles aggregates are significant and is clearly higher in the two first minutes of activation than in the 4 and 6 minute activated pollen grain (Table I). RER: scarce immunolabelling is detected in the mature pollen grain with a maximum in the pollen grain activated for 1 and 2 minutes, and still significant when pollen grain is activated for 4 and 6 minutes. Cytoplasm vesicles: Gold probes are associated with cytoplasm vesicles in all of the activated pollen grain, with significantly more when activation for 1 and 2 minutes than for 4 and 6 minutes. Generative cell: The wall and cytoplasm of the generative cell are labelled significantly and is clearly higher in the two first minutes of activation than in the 4 and 6 minute activated pollen grain. Colloidal gold particles aggregates: Clusters of gold particles are present more extensively in the cytoplasm of the pollen grains activated for 1 and 2 minutes than the one activated for 4 minutes, and associated mainly with amorphous material. No gold probe aggregates are observable at 6 minutes of activation.

Discussion

Diffusible allergenic proteins

The allergenic proteins of *Z. fabago* pollen grains are suddenly released and detected during the first 6 min of activation. After only 1 min of *Z. fabago* pollen hydration, gold particles are already observed in the pollen grain and pollen exudates. This also occurs in the pollen wall of *Betula*, which represents one of the most potent allergen sources in early spring (Grote 1991; Grote et al., 1993, 1999). After 6 min of activation, *Z. fabago* pollen grains show a considerably reduced number of gold particles inside the cytoplasm, in the inner and outer wall as well as in the soluble water pollen exudates. This fact is also comparable to *Betula*, where allergens can be found in the aqueous supernatants after 5 min of rehydration (Grote et al., 1993, 1999).

In *Z. fabago* the rapid diffusion of the allergenic proteins in the activation period is remarkable. This rapidity is higher in *Z. fabago* than observed in *Urticaceae* (Casas et al., 1996) or *Poacea* (Márquez, 1996; Márquez et al., 1992, 1997a, b; Grote et al., 2000, 2001) and is similar to the rate observed in *Betula* (Belin and Rowley, 1971; Grote et al., 1993, 1999). All are considered pollen grains with a high allergenic potential. The rapid diffusion of the allergenic proteins in *Z. fabago* should be related to the significant cytoplasm changes observed after only 1 minute of hydration and with the high velocity of its germination (Castells, 2000). In *Aloe ciliaris*, the cytoplasm changes take place after 20 minutes of the hydration (Ciampolini et al., 1988), and in *Arabidopsis thaliana* changes occur after 10–15 minutes (Van Aelst et al., 1993). In *Z. fabago*, the cytoplasm changes observed during the activation are the transformation of the endomembranes system and the dissociation of the stacks of the cisterns of the RER in the mature pollen grain. Also, the activities of the Golgi apparatus, the mitochondria, the plastids, and the ribosomes are increased. These ultrastructural changes are observed from an inactive state (mature pollen) to an active one (hydration and activation), and last only 6 minutes (Castells, 2000). The increase in respiration activity in *Z. fabago* probably takes place between 2 and 6 minutes of activation indicated by the high proportion of condensed mitochondria observed during this period and finishes with the pollen tube initiation after 6 minutes in the

germinate medium (Castells, 2000). The importance of the mitochondria is related to the rapidity of the germination and it could be comparable with those observed in other pollen grains with high mitochondria development (Hoekstra, 1979).

The two exine layers show an important permeability (Rowley, 1975; Suárez-Cervera and Seoane-Camba, 1986; Suárez-Cervera et al., 1994) and elasticity (Rowley and Skvarla, 2000) to allow the diffusion of allergenic proteins (Knox et al., 1998). The endoporus is a specialised region for germination and the important labelling detected there agrees with the role attributed to this zone (Knox, 1984). The ectocolpus region shows a massive endexine, which is crossed by irregular microchannels in *Z. fabago* (Castells, 2000). The abundant labelling detected in this layer could be related with their permeability. Irregular channels are common in such layer in other taxa (Rowley et al., 1999, 2000). The substructure within the endexine consists of units arranged as short lamellations (Rowley, 1987–88) and the transport of various materials across it has been reported (Rowley and Dunbar, 1970; Rowley and Flynn, 1971). In *Z. fabago* the mesocolpial ectexine is provided with an incomplete tectum, which permits the rapid expelling of glycoproteins. However, in other taxa with complete tectum, such as Cupressaceae (Miki-Hirosige et al., 1994), Urticaceae (Casas et al., 1996) or Poaceae (Márquez et al., 1997a, b), the epitopes of allergenic proteins are detected in the tectum microchannels. The intense labelling observed in the intine agrees with their role and composition (Heslop-Harrison and Heslop-Harrison, 1991).

Role of diffusible proteins

The immunolabelling observed at 1, 2, 4 and 6 minutes of activation should be related with the high level of the antibodies in the sera. The sera were taken from patients who showed positive reaction from pollen provocation test and they presented a specific and high IgE (Belchí et al., 1998). The patients are probably sensitised to different proteins of the pollen grain. The sera antibodies have recognized different epitopes corresponding with proteins, which have been in contact with the patient mucosa. The allergenic protein pool, including the major allergens, released during the contact of the pollen grain with the patients' mucosa, has been recognized. Allergenic proteins located in the pollen exudates may be related to what is needed in the recognition pollen-stigma process. Before hydration, these proteins may be released in the stigma in natural conditions, or in the germination medium in the in vitro germination. On the other hand, pollinosis patients are exposed to atmospheric pollen grains through mucosa. This media will provide pollen grains wet and warm conditions, very similar to that needed to germinate in natural conditions or in an appropriate germination medium. These proteins should be responsible for the pollen-stigma recognition and should indicate a convergence between the mechanisms of the reproductive process and of the proteins release in the sensitised patients mucosa, which have been related in *Parietaria judaica* (Seoane-Camba and Suárez-Cervera, 1986). And because the same or similar biochemical processes take place in the sensitised patients mucosa, it could lead to allergic problems.

The immunolabelling located in the RER and in the Golgi apparatus in *Z. fabago* pollen grains is consistent with their role in the cell membranous systems. The RER with ribosomes in the membrane synthesize new proteins, and some of them migrate to the Golgi apparatus. The Golgi apparatus in the

vegetative cells has two missions: the transfer and the modification of the oligosaccharide chains of the glycoproteins and proteoglycans; and the synthesis of the polysaccharide complexes of the cell wall matrix (Zhang and Staehelin, 1992). The significant labelling in the cisterns of the RER and in the dictyosom vesicles in *Z. fabago* should be consistent with the mission attributed to these organelles. A significant labelling has been also detected in the cisterns of the RER of *Olea europaea* by monoclonal antibodies (Rodríguez-García et al., 1995) and in *Betula* (Grote et al., 1993, 1999; El-Ghazaly et al., 1999). In *Z. fabago* clusters and aggregates of gold particles are present more extensively in the cytoplasm of the pollen grains activated for 1 and 2 minutes than the one activated for 4 minutes, and associated mainly with amorphous material. Clustering of allergens was observed in *Betula* (Grote et al., 1993), *Parietaria* (Casas et al., 1996) and Poaceae (Marquez et al., 1997a, b). In opinion of Grote et al. (1993) that clustering would be a concentration of allergens within preformed tunnels formed in the cytoplasm of the shortly rehydrated pollen grain.

It is not easy to explain the labelling located in the generative cell of *Z. fabago*. The big vegetative cell and the diminutive generative cell have important morphologic and developmental differences. The apparent dimorphism that exists in the cytoplasm volume, and the proteins and RNA contained in it, indicates that these cells have important differences in their genetic makeup and the ability to synthesize proteins (Twell, 1994). These differences have been observed by cytochemical analysis of RNA, and analysis of proteins and histone profiles of purified vegetative and generative cells (Mascarenhas, 1975). We have located significant labelling in both vegetative and generative cells. It is interesting to note the abundant labelling detected in the wall and organelles of the generative cell. The plasmodesmata observed between the generative and vegetative cells of *Z. fabago* (Castells, 2000) might indicate a diffusion of proteins from the generative cell, because of the recognition of their epitopes by the patients' antibodies. Gold particles are also localized in the generative cell of *Lilium* (Nakamura and Miki-Hirosige, 1985), *Cryptomeria* (Miki-Hirosige et al., 1994), and *Betula* (Grote et al., 1999). This fact should indicate that both cells, the vegetative and generative cells are involved in the pollen-stigma processes.

Conclusion

The rapid activation and release of the allergenic proteins appears to be the main cause of the strong allergenic activity of *Z. fabago* pollen grains. In our opinion, due to the allergenic potential of its pollen grains, *Z. fabago* should be considered in aeropalynological and clinical studies in the arid zones of the Mediterranean region. Because *Z. fabago* is an invasive plant, it can be found far away from its natural habitat, and this should further increase the interest in the consequences of the high allergenic potential shown by *Z. fabago*.

Acknowledgements. The authors are grateful to the Scientific Technical Services at the University of Barcelona for their careful preparation of sections for TEM in this study. We are especially grateful to Dr. Peter Scheff (University of Illinois at Chicago) for his careful revision of our English. This study was supported by Grant PB96–0393, Ministerio de Educación y Ciencia, Spain.

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