



Changes in the *Plantago lanceolata* mature pollen ultrastructure and Pla l 1 allergen localisation after grain hydration

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Abstract

We have conducted the study of ultrastructural changes of wall and cytoplasm of *Plantago lanceolata* (English plantain) pollen grains during the first 10 min of hydration and activation processes, prior to germination, and localisation of Pla l 1, the major allergen of these pollen grains with immunocytochemical methods. The samples were fixed using conventional and freezing protocols for transmission electron microscopy. During the activation process, the intine is thickened in the apertural region and cytoplasm undergoes changes in the number of lipid bodies, amyloplasts, vacuoles and ribosomes. Also, we observed an association between lipid bodies, cisternae of rough endoplasmic reticulum, dictyosomes and vacuoles. An increase in the presence of allergenic particles (Pla l 1) in the exine, intine and the cytoplasm in activated pollen grains was detected, whereas this presence is not significant in mature pollen grains. The increase in the production and release of this allergen when pollen grains are activated suggest that Pla l 1 has an important role in pollen–stigma recognition and in the subsequent development of the pollen tube.

Keywords: *Plantaginaceae, activated pollen, ultrastructure changes*

Most allergens in the pollen grains are soluble proteins which have enzymatic activities that are essential for plant cell development (Radauer & Breiteneder 2006). It is well known that, during the *in vitro* germination of pollen, a huge number of proteins are overexpressed and released into the germination medium (Sheoran et al. 2009). Allergens are stored inside the pollen grain (cell wall and protoplasm) and diffuse during germination (Belin & Rowley 1971). These soluble proteins play a significant role in pollen–stigma recognition, formation of pollen tube and its passage through the style so fertilisation may take place (Castells et al. 2002; Vega-Maray et al. 2006a, 2006b; Suárez-Cervera et al. 2008; Songnuan 2013; Chen et al. 2016). During that activation, prior to germination processes, the pollen grains undergo a series of changes. The main changes are related to the pollen wall architecture; the number and degree of development of the organelles; the amount and nature of reserve substances; the gen-

erative cell composition and its association with the nucleus of the vegetative cell (Ciampolini et al. 1988; Márquez et al. 1997a, 1997b; Suárez-Cervera et al. 2003; Vega-Maray et al. 2003).

The pollen allergens have been the subject of immunochemical research for several decades. Allergens have been isolated and their physical, as well as their immunochemical characteristics, have been determined (Calabozo et al. 2001; Castro et al. 2007). Localisation of pollen proteins by immunoelectron microscopic methods has been the aim of several research groups. There are therefore studies about immunolocalisation in different allergenic species, such as, *Acacia saligna* (Labill) H. L. Wendl. and *Acacia victoriae* Benth. (Iriam et al. 2013); *Artemisia* spp. L. (Gao et al. 2019); *Betula pendula* Roth. (Emilson et al. 1996; El-Ghazaly et al. 1999); *Cupressus arizonica* Greene. and *C. sempervirens* L. (Suárez-Cervera et al. 2003; Canini et al. 2004); *Eucalyptus globulus* Labill. (Eliseu & Dinis 2008); *Frax-*

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inus angustifolia Vahl. (Rodríguez-Rajo et al. 2010); *Olea europaea* L. (Alché et al. 1999; Castro et al. 2003; Morales et al. 2008); *Parietaria judaica* L. (Vega-Maray et al. 2004, 2006a); *Plantago lanceolata* L. (Castro et al. 2007); *Platanus acerifolia* (Aiton) Wild. (Suárez-Cervera et al. 2005); *Poaceae* Barnhart. (Márquez et al. 1997a, 1997b; Grote et al. 2000) and *Urtica dioica* L. (Vega-Maray et al. 2006b).

The genus *Plantago* is very common in nitrified areas such as roadsides. These plants have anemophilous pollination with a high pollen production (González-Parrado et al. 2015). It has reduced corollas and stamens with developed filaments that expose the anthers to the action on the wind (Van Damme 1992). *Plantago lanceolata* pollen is spheroidal, pantoporate, small (10–25 µm), operculate (with annulus) and verrucate, microechinate, perforate ornamentation.

Plantago lanceolata pollen is a major cause of pollinosis in temperate zones (Watson & Constable 1991; Calabozo et al. 2002; Huertas et al. 2003). The incidence of allergy to *P. lanceolata* has been described in Australia (Krilis et al. 1985) with an incidence of 19% and Europe: Italy with a 7% of prevalence (D'Amato & Lobefalo 1989); Austria with 36% (Gadermaier et al. 2014); Portugal with an incidence of 31% (Sousa et al. 2014). High prevalence of allergy to this pollen has been described in Spain, with percentages close to 8% (García-González et al. 1998), 44.1% (González-Parrado et al. 2014) and 32% (Subiza et al. 1995). Several authors observed that Pla l 1, the major allergen of *P. lanceolata* pollen grain, consisted of a hydrophilic and polymorphic allergen with a polypeptide chain of 131 amino acid residues. It can occur in different forms: unglycosylated (17 kDa), glycosylated (23 kDa) and dimeric (32–39 kDa) (Calabozo et al. 2001; Castro et al. 2007; Sousa et al. 2014).

Major olive pollen allergen (Ole e 1) shares sequence identity and cross-reactive epitopes with Pla l 1 (Castro et al. 2007; Sousa et al. 2014; Iglesias-Otero et al. 2015). The structural similarities and similar localisation between Ole e 1 and Pla l 1 might account for a shared biological function in the pollen grain (Castro et al. 2007).

Although members of Ole e 1 family show sequence similarity with soybean trypsin inhibitor-related proteins, no conclusive evidence of this putative relationship has been established to date (Castro et al. 2007). Songnuan (2013) pointed out that members of the Ole e 1-related proteins may play a role in pollen tube guidance.

In the present study, ultrastructure and cytochemistry of pollen from mature pollen before another release (mature pollen) and after hydration (activated pollen) of *Plantago lanceolata* were investigated using both chemical fixation and rapid freeze-fixation. The

aim is to gain a detailed knowledge of the structural organisation of the pollen of English plantain to better understand its physiology and its performance during shedding, storage and germination. It is the first study about ultrastructural changes and immunolocalisation of Pla l 1 in activated pollen of *P. lanceolata*.

Material and methods

Plant material

Twenty plants of *Plantago lanceolata* were collected in the surroundings of the University of León (Spain) during the months of May and June. The collection area was an abandoned land. Mature pollen grains were released from the anthers by vigorously shaking the inflorescences. The pollen samples were used fresh, without any storage.

In vitro hydration and activation

Initially, we conducted a test in order to obtain the activation times to use in this work since pollen grains are activated before they form the pollen tube. To that end, pollen grains of *Plantago lanceolata* were deposited in agar germination medium which was incubated in a wet-chamber that was observed every 5 min with an optic microscope. In this way, we could verify that most of the pollen grains formed the pollen tube in about 15 min.

Once the time taken to germinate the pollen grains was known, hydration and activation of mature pollen grains were induced *in vitro* by sprinkling them onto 0.7% pure agar germination medium (Walden 1993) for 5 and 10 min, at room temperature to generate humidity and temperature conditions similar to receptive stigma. After these times, the process was interrupted, and pieces of agar with pollen grains were cut and fixed.

Transmission electron microscopy (TEM)

Due to difficulties in preserving the pollen grain ultrastructure, different protocols were used for studying the ultrastructure and localisation of Pla l 1 in pollen grains.

Conventional method using Spurr's resin. — Dehiscent anthers and *in vitro* hydrated and activated pollen grains were fixed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde buffered by 0.1 M cacodylate, pH 7.3 for 2 h at 4 °C. Later, the samples were washed in 0.1 M cacodylate buffer, pH 7.3, for 40 min and post-fixed with 1% osmium tetroxide in 0.8% phosphate-buffered potassium ferricyanide ($K_3Fe(CN)_6$) at pH

7.3, for overnight at 4 °C. This was followed by dehydration in an acetone series and embedding in Spurr's resin. Sections of the blocks containing the samples were obtained with a Reichert-Jung Ultracut E microtome. The sections were mounted on gold grids.

For immunolocalisation, ultrathin sections of pollen grains were incubated at room temperature on drops of 5% bovine serum albumin (BSA) in 0.1 M phosphate-buffered saline (PBS) for 15 min, followed by monoclonal antibody Pla 1 1 (Abelló, Madrid, Spain), using a 1:10 dilution in PBS 0.1 for 12 h. After four 20 min washes with 0.25% PBS in Tween 20, the sections were incubated for 1 h with a goat anti-mouse immunoglobulin G (IgG) antibody coupled to 18 nm gold particles (BioCell International, Austria), diluted 1:50 in PBS. Then sections were washed with PBS and ultrapure water. Sections were stained with uranyl acetate and lead citrate and observed in a JEOL 1010 electron microscope (Tokyo, Japan).

Freeze-substitution for immunolabelling. — Mature and *in vitro* hydrated pollen grains were fixed using freezing protocols for transmission electron microscopy (TEM). Samples were chemically fixed by immersion at 4 °C with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After washing with the same buffer containing 50 mM glycine, samples were gradually infused in 30% glycerol in the same buffer, as cryoprotection, and they were frozen in liquid propane at -190 °C (Leica EM CPC, Vienna, Austria) and stored in liquid nitrogen at -196 °C until the freeze-substitution was done. Freeze-substitution was performed in an automatic freeze substitution (AFS) system (Leica), using methanol containing 0.5% uranyl acetate, for 3 days at -90 °C. On the fourth day, the temperature was slowly increased, by 5 °C/h, to -50 °C. At this temperature, samples were rinsed in methanol, and then infiltrated and embedded in Lowicryl HM20 for 10 days. Ultrathin sections were obtained using a Leica Ultracut UCT ultramicrotome and were picked up on Formvar-coated gold grids.

For immunogold labelling, samples obtained by cryosections were incubated at room temperature on drops of 0.1 M PBS for a few seconds, followed by 0.05 M glycine in 0.1 M PBS for 8 min and 0.1 M PBS for a few seconds again. The samples were washed three times for 10 min each with 5% BSA and one fast washing with 1% BSA. Subsequently, the sections were incubated with monoclonal antibody Pla 1 1 dilute 1/15 in 0.1 M PBS and 1% BSA for 2 h. After five washes with drops of 0.25% Tween 20 in 0.1 M PBS and a quick wash with 1% BSA in 0.1 M PBS, sections were incubated for 1 h using a goat anti-mouse IgG antibody coupled to 12 nm gold particles (BioCell

International), using a dilution 1/40 in 0.1 M PBS and 5% BSA. After that, sections were washed with PBS and ultrapure water. Sections were stained with uranyl acetate and lead citrate and observed in a JEOL 1010 electron microscope.

In order to determine the frequency of gold particles present in different cellular structures, the number of gold particles in each organelle or surface was counted and its area was calculated. In this way, the frequency of colloidal gold particles per square micrometre could be calculated. For each structure, six or more were counted.

Moreover, the measurement of different organelles has been carried out with the 'Fiji' program. The length and diameter of mitochondria, lipids and starches were measured. In addition, the thickness of the different layers of the pollen grain wall (exine and intine) in mature pollen and in activated pollen for 5 and 10 min was measured. Furthermore, the number of endoplasmic reticulum and dictyosomes cisternae was counted. Fifteen measurements of each structure were taken, except for the wall of the apertural region where only six measurements were taken due to the difficulty of capturing microscopical images of this zone.

Results

Ultrastructure

Mature pollen grains. — *Plantago lanceolata* pollen grains have a big vegetative cell and two sperm cells, although most of the time we observe only one sperm cell (Figures 1, 5C, D).

The ultrastructure of the ectexine shows a complete and wide tectum, short and irregular shape collumelae and a fragmented foot layer. Endexine is discontinuous and compacted. The intine is thicker than endexine (Figure 2A, B).

Operculated pores with annulus were observed in the apertural sporoderm (Figure 2A).

The vegetative cell cytoplasm contains the organelles and necessary material for germination and growth of pollen tube. The lipid bodies are numerous, small (0.75 µm/0.59 µm), spherical or elongate (Figure 1, Table I). It can be present anywhere inside the vegetative cell cytoplasm such as the vacuoles and both of them appear fused in many cases (Figures 1, 2A). Starch granules are present in a variable number, have a medium size (1.19 µm/0.7 µm) (Table I) and are usually surrounded by endoplasmic reticulum (Figures 1, 2A, 5A, B). Rough endoplasmic reticulum is abundant and with several cisternae together, although isolated cisternae are also observed (mean 2.1 cisternae, Table I). We observe a clear relationship between

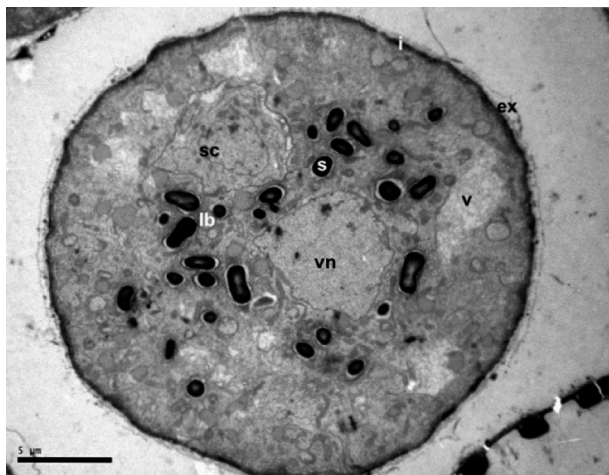


Figure 1. Section of mature pollen grain of *Plantago lanceolata* obtained with conventional protocol. Abbreviations: ex, exine; i, intine; lb, lipid bodies; s, starches; sc, sperm cell; v, vacuoles; vn, vegetative nucleus.

this organelle and dictyosomes (Figure 2C). Dictyosomes are few in number and are distributed throughout the cytoplasm. Usually, consist of 4, 5 or 6 (mean 4.87 cisternae) short cisternae arranged in parallel (Figure 2C, Table I) and surrounded by numerous, small and spherical vesicles (Figure 2C). Mitochondria are spherical (Table I); abundant; have numerous irregular shaped cristae and are located mainly in the cell periphery, specifically in the mesopore (Figures 2C, 5A, B). There are numerous ribosomes, scattered throughout the cytoplasm and attached to the cisternae of rough endoplasmic reticulum (Figure 2A).

The nucleus is large and has an irregular shape (Figures 1, 2D). Usually, it is located in the centre of the vegetative cell and close to the spermatogenic cells (Figures 1, 2D). The nucleus is in an interphase state and is heterochromatic. It has large areas of low electro-density where chromatin is poorly condensed (euchromatin) and high electro-density few granules, where it condensed the chromatin (heterochromatin).

Spermatogenic cells are small and are situated near vegetative nucleus (Figures 1, 2D). Their cell wall is undulating and has two membranes (Figure 5D). In longitudinal section, spermatogenic cell is long and sometimes we can observe a cytoplasmic extension in the form of a tail (Figures 2D, 5D). In transverse section the central region of this cell has a rounded contour. The cytoplasm is reduced but we can observe organelles such a mitochondria and vacuoles. The sperm nucleus occupies almost completely the cell (Figures 1, 2D, 5D).

Activated pollen grains. — During the period of activation, the pollen grain has undergone several modifi-

cations. After 5 min in culture medium, lipid inclusions and amyloplasts have increased in number with respect to the mature pollen grain. Most of the lipid inclusions appear near the amyloplasts and rough endoplasmic reticulum (Figure 3). However, after 10 min of activation periods the number of lipid inclusions decrease. The amyloplasts are one of the most abundant organelles in this state, together with ribosome, they also increase.

In the apertural region the sporoderm presents a slight thickening of the exine and intine (Figure 4C, Table I).

The mesopore has not undergone major changes from the mature pollen grain (Figure 6A, Table I).

During the activation period, the number of vacuoles decreases and those that exist are attached to lipid bodies (Figure 3). Mitochondria appear near the vegetative nucleus, spermatogenic cells and apertural area and have more elongated and less numerous than the mature state (Figures 6C, D, 7A, Table I).

In the first 5 min of activation, the rough endoplasmic reticulum cisternae appear more than in mature pollen, but are usually shorter (Figure 4A, B). Many saccules of rough endoplasmic reticulum occur in the periphery of the vegetative cytoplasm, releasing its contents into the intine. After 10 min of activation, both of them increase in number and also length (Figures 4C, D, 7B, C).

The changes in dictyosomes are observed at 5 and 10 min, its number increases and most of them have cisternae arranged concentrically (Figures 4B, 7A–C). Additionally, at this time we observed a large amount of dictyosomal vesicles throughout the vegetative cell cytoplasm and especially in the vicinity of the wall, reaching to fuse with plasma membrane (Figures 4B, 7A–C).

The nucleus and spermatogenic cells have not changed; its ultrastructure is similar to those observed in mature pollen grains (Figures 3, 6C, D, 7D).

Immunocytochemical

Equivalent immunolabelling patterns were obtained with the two protocols used.

Mature pollen grains. — Scarce and no significant labelling was observed in the pollen wall (exine and intine). We have found some labelling in all organelles except for mitochondria (Figures 5A, B, 8). In dictyosomes; endoplasmic reticulum and in starch granules the labelling is more numerous than in the rest of organelles (Figures 5A, B, 8).

We did not detect labelling in the nucleus and in sperm cells (Figure 5C, D).

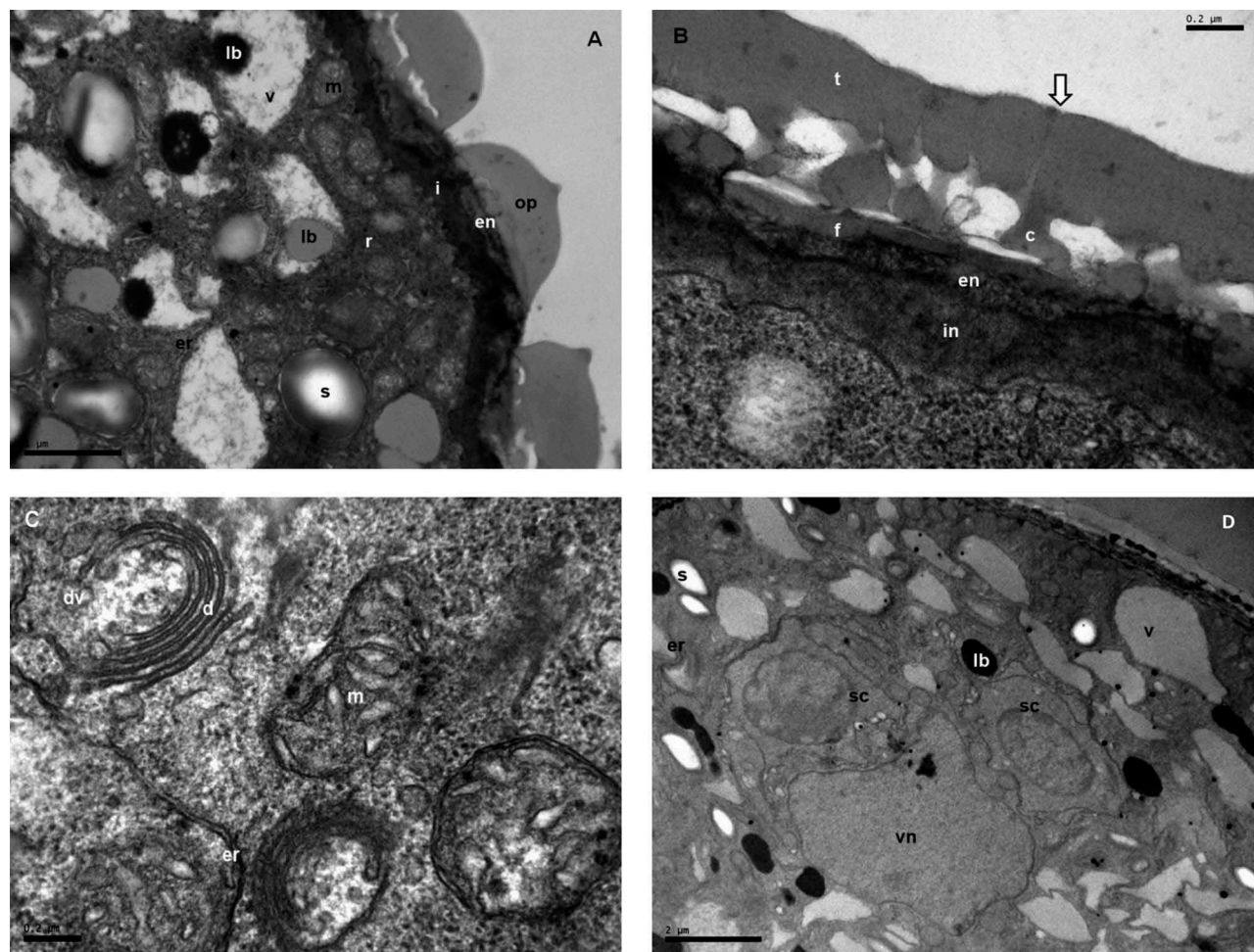


Figure 2. Ultrastructure of mature *Plantago lanceolata* pollen grain (conventional protocol). **A.** Apertural area. **B.** Pollen wall. **C.** Cytoplasm. **D.** Sperm cells around the vegetative nucleus. Abbreviations: c, columella; d, dictyosome; en, endexine; er, rough endoplasmic reticulum; f, foot layer; i, intine; lb, lipid bodies; m, mitochondria; op, opercula; r, ribosomes; s, starches; sc, sperm cells; t, tectum; v, vacuoles; vd, dictyosomal vesicles associated to cisternae of rough endoplasmic reticulum and (arrows) microchannels.

Table I. Mean and standard deviation of the thickness of different layers of the pollen grain wall; length and diameter of mitochondria, amyloplasts and lipid bodies; number of endoplasmic reticulum and dictyosomes cisternae.

		Activated pollen grains		
		Mature pollen	5 minutes	10 minutes
Mesopore zone	Exine	0.63 ± 0.15	0.66 ± 0.18	0.63 ± 0.21
	Intine	0.33 ± 0.12	0.39 ± 0.10	0.31 ± 0.10
Apertural region	Exine	0.75 ± 0.12	0.99 ± 0.08	0.81 ± 0.28
	Intine	0.35 ± 0.09	0.71 ± 0.01	0.40 ± 0.11
Mitochondria		0.51 ± 0.12/0.4 ± 0.08	0.56 ± 0.15/0.41 ± 0.06	0.57 ± 0.12/0.40 ± 0.11
Amyloplast		1.19 ± 0.39/0.70 ± 0.20	1.18 ± 0.41/0.71 ± 0.13	1.34 ± 0.50/0.87 ± 0.17
Lipid bodies		0.71 ± 0.19/0.59 ± 0.20	0.87 ± 0.14/0.66 ± 0.12	0.73 ± 0.19/0.53 ± 0.10
Endoplasmic reticulum		2.1 ± 0.74	2.2 ± 0.77	2.5 ± 0.92
Dictyosome		4.87 ± 1.06	4.33 ± 0.98	4.67 ± 1.18

Activated pollen grains. — After 5 min in culture medium, gold colloidal particles were more numerous in the exine and intine (Figures 6A, 8). Labeling appeared in the exterior of the pollen grains (Figure 6A). We detected an increase in the number

of gold colloidal particles in starches (Figures 6B, 8). In the rest of the cytoplasm organelles differences with regard to the mature pollen were not observed. In the sperm cell and vegetative nucleus some gold marks existed (Figure 6C, D).

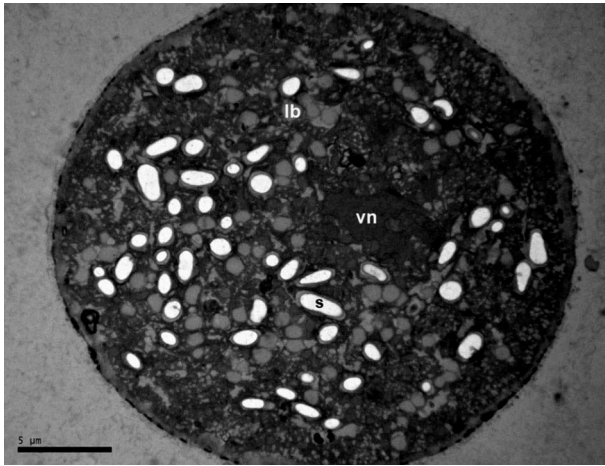


Figure 3. Section of *Plantago lanceolata* activated 5 min (conventional protocol). Abbreviations: lb, lipid bodies; s, starches; vn, vegetative nucleus.

After 10 min of hydration, we found a light increase in the labelling in vegetative nucleus and sperm cells (Figure 7D), mainly in starch granules; vacuoles; lipid bodies; rough endoplasmic reticulum and in Golgi stacks as well as associated vesicles (Figures 7A–C, 8). Moreover, gold colloidal particles were discerned in the external medium.

No significant labelling was observed in controls.

Discussion

Technical difficulties in preserving the pollen grain ultrastructure are a likely reason for the scarcity of studies in this field of research within the Plantaginaceae. Only an ultrastructure study on pollen wall and tapetum development in *Plantago major* was found (Gabarayeva et al. 2017). So far, the present work is the first one dealing with the changes in the

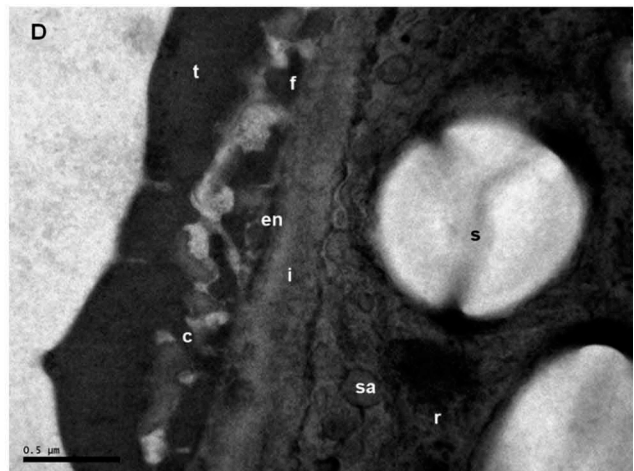
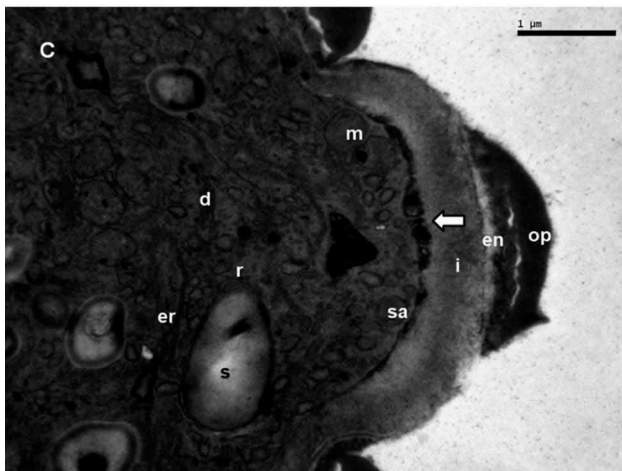
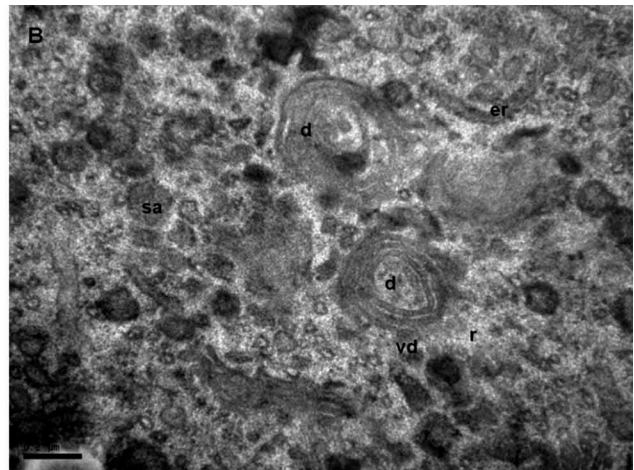
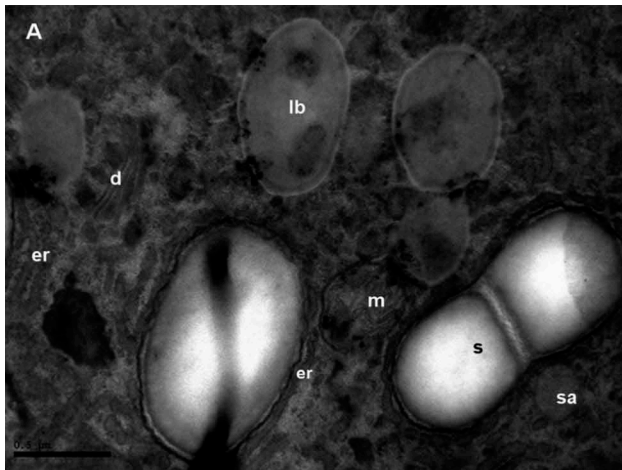


Figure 4. Ultrastructure of activated *Plantago lanceolata* pollen grains (conventional protocol). **A, B.** Cytoplasm of activated *P. lanceolata* pollen during 5 min. **C.** Detail of apertural zone in pollen activated 10 min. **D.** Wall and cytoplasm of activated *P. lanceolata* pollen grains during 10 min. Abbreviations: c, columella; d, dictyosome; en, endexine; er, rough endoplasmic reticulum; f, foot layer; i, intine; l, lipid bodies; m, mitochondria; op, operculum; r, ribosomes; s, starches; (arrow) saccule some of them are fusing with plasma membrane, sa, saccule; t, tectum; vd, dictyosomal vesicles.

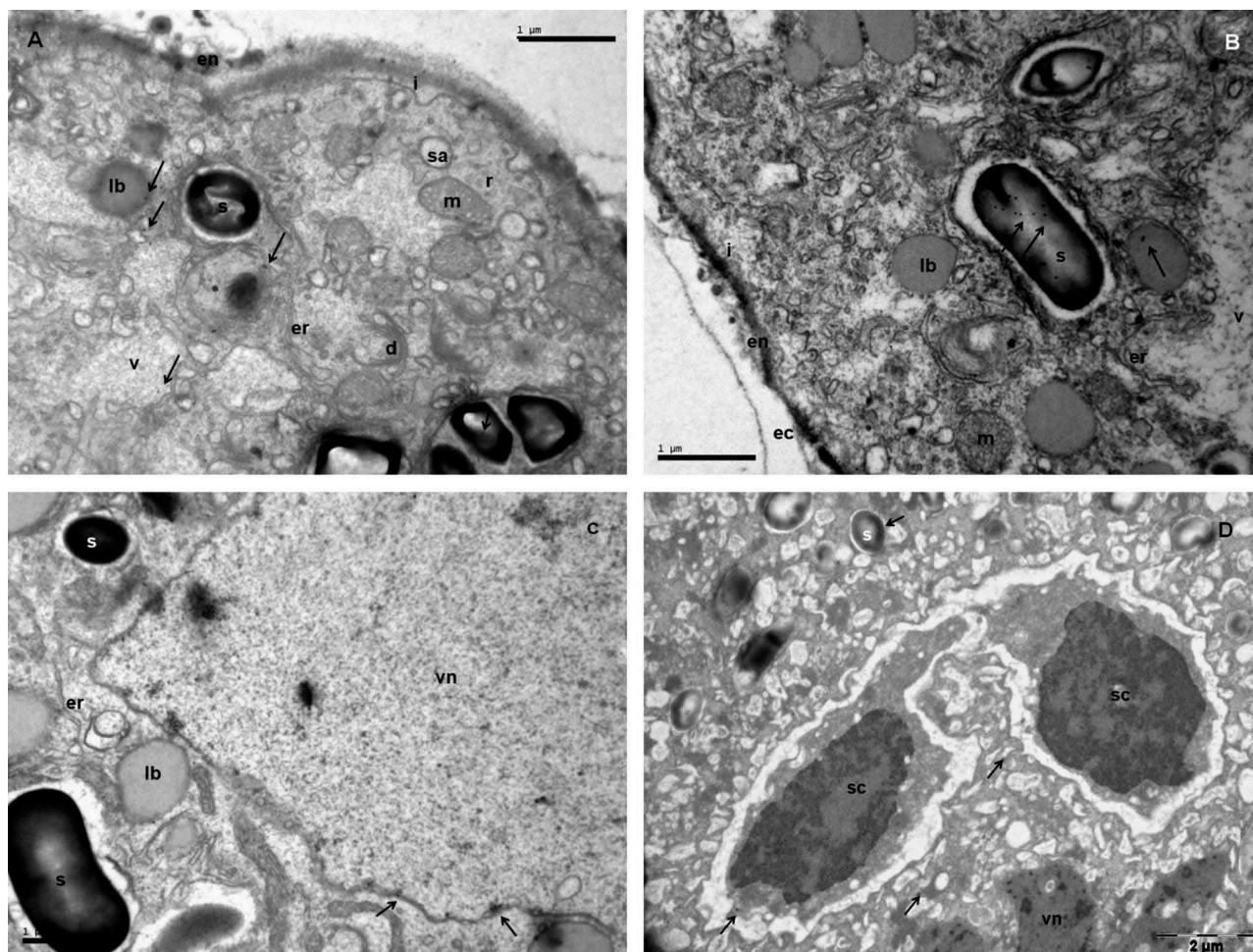


Figure 5. Immunocytochemical localisation of Pla 1 in mature *Plantago lanceolata* pollen grain section. **A.** Apertural zone with scarce labelling (arrows) with conventional protocol. **B.** Mesopore zone and cytoplasm. Some labelling (arrows) is observed in starches and lipid bodies with conventional protocol. **C.** Detail of nucleus of vegetative cell with conventional method in which exists labelling (arrows) although this labelling is not abundant. **D.** Freezing protocol. Detail of sperm cells in which we can perceive nucleus and the cytoplasm of the cells which are particles of colloidal gold (arrows). Around the sperm cells observed the vegetative cell nucleus and starches. Abbreviations: d, dictyosomes; ec, ectexine; en, endexine; er, rough endoplasmic reticulum; i, intine; lb, lipid bodies; m, mitochondria; r, ribosomes; s, starches; sa, sacculles; sc, sperm cells; v, vacuole; vn, vegetative nucleus.

organisation of the *P. lanceolata* pollen during the activation process.

We have found differences between the two methods used. The preservation of the wall ultrastructure and the cytoplasmatic organelles with the Spurr resin method have been quite good, while with the freeze-substitution method, many of the organelles are not observed clearly. No significant differences were found in labelling with Pla 1 between the two methods. Other authors have obtained good results with the Spurr resin method in pollen of grass (Márquez et al. 1997a, 1997b); *Euphorbia peplus* (Suárez-Cervera et al. 2002); *Urtica dioica*, *Parietaria judaica* (Vega-Maray et al. 2006a, 2006b) and in a study about the endexine in several species (Weber & Ulrich 2010).

Freeze-substitution has been used by El-Ghazaly et al. (1999) in an immunocytochemical study of *Betula* pollen, by Alché et al. (1999, 2002) in the localisation of Ole e 1 in olive pollen grains, by Suárez-Cervera et al. (2005) in the study of allergenic proteins Pla a 1 and Pla a 2 in *Platanus acerifolia* and by Rodríguez-Rajo et al. (2010) in *Fraxinus angustifolia*. According to Alché et al. (1999), the cryosubstitution methods preserve the proteins better in immunocytochemical analysis.

Modifications have been described in pollen grains during the activation period, at different times, in *Zygophyllum fabago* at 2 min (Castells et al. 2002); in *Cupressus arizonica* and *C. sempervirens* at 5 min (Suárez-Cervera et al. 2003); in *Parietaria judaica* in the first 10 min (Vega-

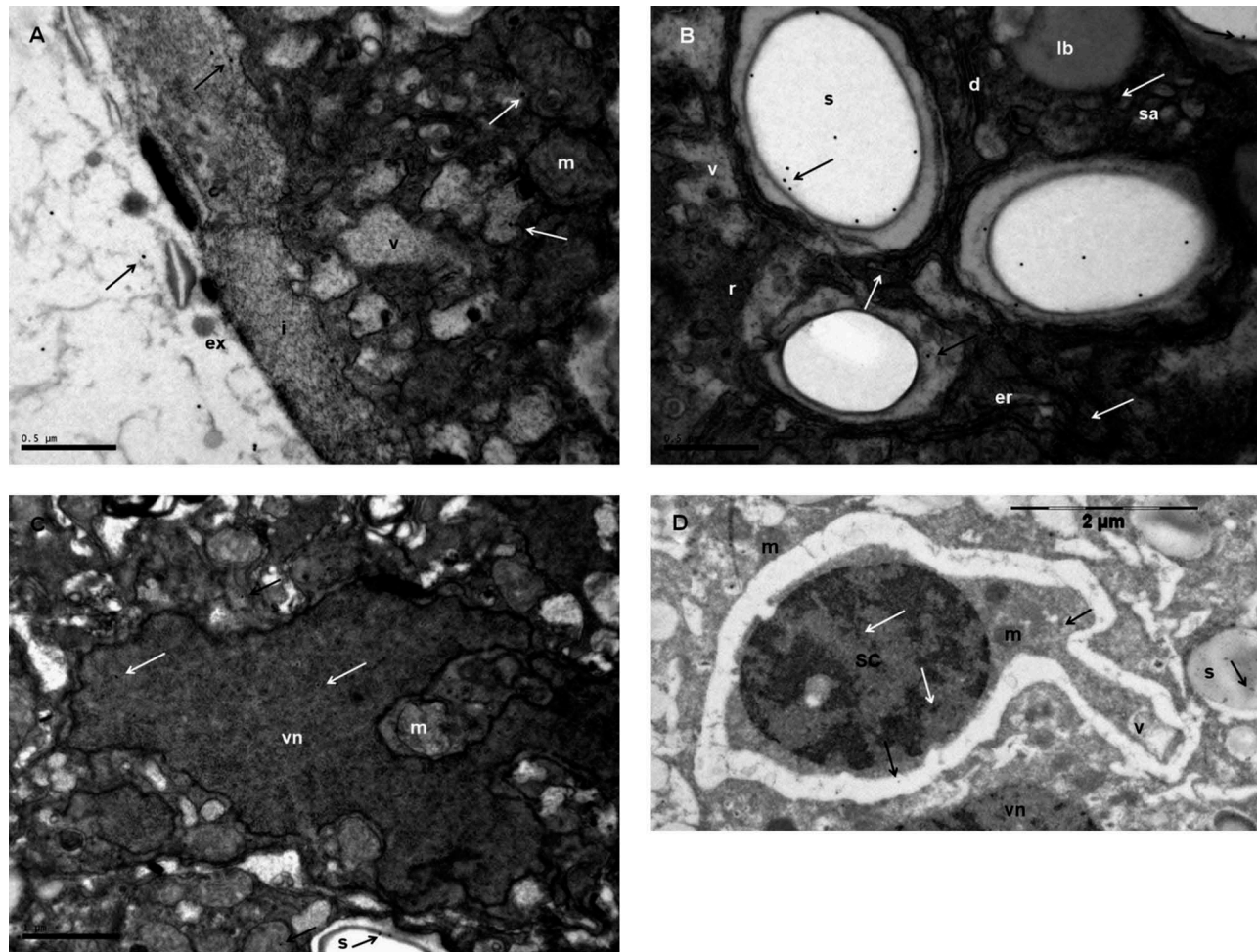


Figure 6. Immunocytochemical localisation Pla 1 in *Plantago lanceolata* pollen grain after 5 min in germination medium. **A.** Detail of cell wall with conventional protocol. **B.** Detail of cytoplasm of pollen grain using a conventional protocol. **C.** Detail of nucleus of vegetative cell with conventional method. **D.** Detail of sperm cell with freezing protocol. Abbreviations: d, dictyosomes; ex, exine; er, endoplasmic reticulum; i, intine; lb, lipid bodies; m, mitochondria; r, ribosomes; s, starches with labelling (arrows); sa, saccules or vesicles associated to endoplasmic reticulum; sc, sperm cell; v, vacuoles; vn, vegetative nucleus.

Maray et al. 2003); in *Arabidopsis thaliana* occurs within 10 to 15 min (Van Aelst et al. 1993), and *Aloe ciliaris* (Ciampolini et al. 1988) at 20 min, so it is in *Prunus dulcis* (Arcalís 2001). In *Plantago lanceolata* these changes began in the first 5 min of the occurrence of hydration.

We observe more pollen grains of *Plantago lanceolata* with one sperm cell than pollen grains with two sperm cells. It could be due to a possible coexistence between pollen grains bicellular and tricellular in this taxon. The simultaneous presence of bicellular and tricellular pollen grains have been described in some taxa of Magnoliidae (Sampsom 1969; Gardner 1974), *Euphorbiaceae* (Webster & Rupert 1973) and *Oleaceae* (Johri et al. 1992). According to Lora et al. (2009), the temperature during the final phases of pollen grain development modulates the proportion of one or other type of pollen grain in *Annona cher-*

imola. Other authors pointed out that the adaptive advantage of tricellular pollen grains is the fast germination (Brewbaker 1967; Mulcahy & Mulcahy 1988). However, the fast germination is a characteristic that we can observe in *P. lanceolata*. The pollen tube is formed within 15 min of activation in *P. lanceolata* pollen grains (personal observation). Williams et al. (2014) observed that tricellular pollen is often found in species with extreme, time constrained lifestyles. Maybe the bicellular and tricellular pollen grains of *P. lanceolata* would depend on the environmental conditions in which the plant has grown.

During the activation process, in *Plantago lanceolata* we detected a thickening of the endexine and intine in the apertural area, the apertural membrane ruptured, and the operculum separated from the apertural margins. Some researchers have found

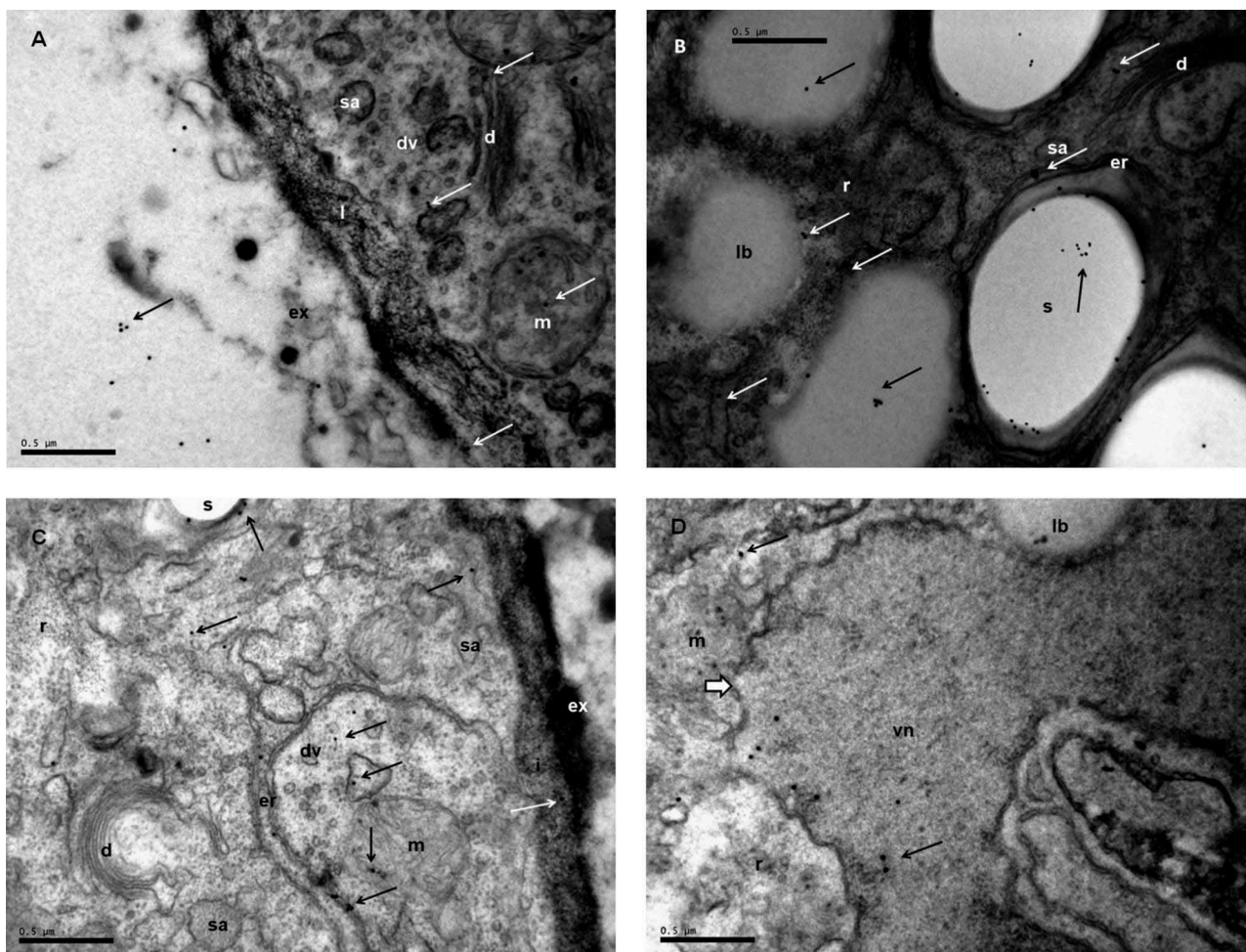


Figure 7. Immunocytochemical localisation Pla 1 in *Plantago lanceolata* pollen grain after 10 min in germination medium with conventional protocol. **A.** Detail of wall cell where observed the exine and intine and abundant particles of colloidal gold in the material exuded (arrows). **B.** Detail of lipid bodies and starches with abundant labelling (arrows). **C.** Cytoplasm of the cell. **D.** Nucleus of vegetative cell which has numerous pores in its membrane. Abbreviations: d, dictyosome; dv, dictyosomal vesicles; er, endoplasmic reticulum; ex, exine; i, intine; lb, lipid bodies; m, mitochondria; r, ribosomes; s, starches; sa, saccules associated to endoplasmic reticulum; vn, vegetative nucleus.

similar modifications in grass pollen at the same time (Márquez et al. 1997a) and *Euphorbia peplis* (Suárez-Cervera et al. 2002). Moreover, in the mature pollen grain of *P. lanceolata* we did not observe labelling in the nonapertural and apertural zone. Similar results were found by Castro et al. (2007) in *P. lanceolata*. However, labelling appears in the exine, intine and the exudates during the activation process. These results are similar to the results obtained by other authors, such as Gao et al. (2019) in *Artemisia*, Grote et al. (1993) in *Betula*, Alché et al. (2004) in *Olea europaea*, Castells et al. (2002) in *Zygophyllum* Vega-Maray et al. (2004, 2006a, 2006b) in *Urticaceae*.

We located allergenic proteins in the material exuded from the pollen grains because the exine layer allows the diffusion of allergenic proteins through the microchan-

nels of the exine (Rowley 1975; Seoane-Camba & Suárez-Cervera 1986; Knox et al. 1998).

The trafficking of proteins through the intine confirms the functions attributed to this layer in the process of pollination and fertilisation (Heslop-Harrison & Heslop-Harrison 1991; Suárez-Cervera et al. 2000).

Pollen grains activated for 5 min have an increase in the number of lipid bodies, becoming one of the most abundant organelles. The increased presence of lipid bodies in activated pollen grains has been highlighted by Arcalís (2001). During the activation and germination process, lipid spherosomes are the primary energy reserve. For this reason, we detected a decrease in the number of lipid inclusions after 10 min of activation. This, we believe, may be related to the conversion of lipids to polysaccharides

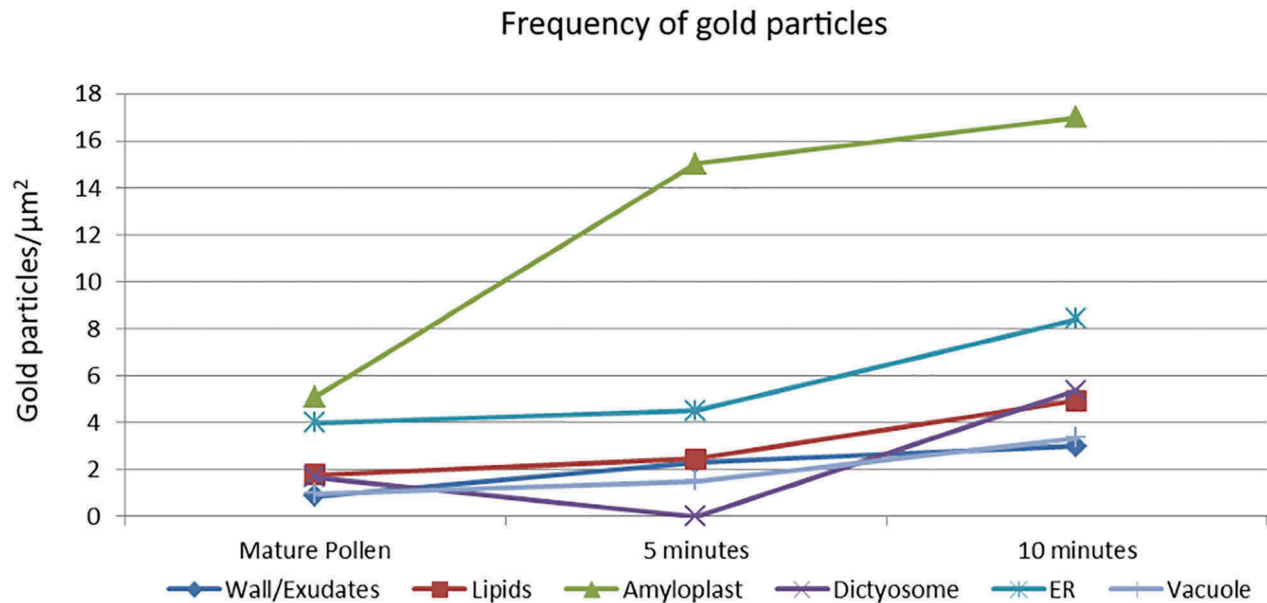


Figure 8. Frequency of gold particles in wall/exudates, lipids, amyloplasts, dictyosomes, endoplasmic reticulum (ER) and vacuoles in mature pollen grains and activated pollen.

through the fusion of lipid and vacuoles. This fact was also proposed by Castells (2000) and Vega-Maray et al. (2004).

We have noticed a clear association between lipids, amyloplasts and cisternae of rough endoplasmic reticulum, so we may assume that, within the vegetative cell of the pollen grain occurs the transformation of lipids to polysaccharides through an endomembrane system. The same association was noted by Castells (2000) and Vega-Maray (2002). Weber (1989) pointed out that low molecular weight carbohydrates are transported by rough endoplasmic reticulum vesicles to the dictyosomes, where they are synthesised to complex polysaccharides.

Plantago lanceolata has numerous amyloplasts. Many of them are surrounded by endoplasmic reticulum cisternae. This has been observed by other authors such as Castells (2000) in *Zygophyllum fabago* and Vega-Maray (2002) in *Parietaria judaica*. According to Vega-Maray (2002), this may be because endoplasmic reticulum cisternae are involved in starch hydrolysis during pollen activation and germination.

There are both lipids and starches in activated pollen as reserve substances, but the amount of lipids is smaller. The coexistence of two reserve substances has been indicated by other authors in *Zygophyllum fabago* (Castells, 2000), in *Prunus dulcis* (Arcalís 2001) and in *Parietaria judaica* (Vega-Maray 2002). However, Vardar et al. (2013) denoted that the cytoplasm of mature pollen of *Asphodelus aestivus* was filled with starch grains but lacked lipid.

We found gold markers in the lipid bodies and mainly in starches in mature and activated pollen grains. However, Castro et al. (2007) observed gold particles occasionally surrounding starch granules in mature pollen. Several authors had detected starches with labelling during the activation process (Knox & Suphioglu 1996; Márquez et al. 1997a; El-Ghazaly et al. 1999). Pacini et al. (1992) indicated that if the material is manipulated under anhydrous conditions, labelling does not appear in starches, whereas if the material is in contact with an aqueous medium, the labelling is evident, since humidity seems to trigger the activation of certain allergens that mobilise the energy reserves during germination. Behrendt and Becker (2001) observed that group 5 allergens of grass pollen are predominantly mapped to the amyloplasts, but when they used strictly anhydrous fixation protocols this allergen was not present in starch granules.

Labelling in vacuoles is similar in mature and activate pollen grains of *Plantago lanceolata*. This labelling can be due to the function of storage of proteins, mineral salts and other substances. Vacuoles with gold markers have been discovered in other activated pollen grains, such as *Urticaceae* (Casas et al. 1996; Vega-Maray et al. 2006a, 2006b). However, vacuoles without labelling were found by Márquez et al. (1997a) in grasses, Alché et al. (2002) and Castro et al. (2003, 2007) in *Olea europaea* and Castro et al. (2007) in *P. lanceolata*.

The change in the morphology of mitochondrial cristae may mean the transition from an inactive to an active state (ortodoxe conformation to condensed

conformation) which increased respiratory. This fact has been detected in other pollen grains (Dickinson 1967; Southworth & Dickinson 1981; Castells 2000).

There is an increase in the number of rough endoplasmic reticulum cisternae, which means more protein production, as the rough endoplasmic reticulum is closely related to the synthesis and secretion of these molecules. In mature pollen grains during activation, packages of cisternae disappear and the cisternae are scattered throughout the cytoplasm. This has been perceived in other pollen grains of *Nicotiana glauca* (Cresti et al. 1985), *Aloe ciliaris* (Ciampolini et al. 1988), *Papaver dubium* (Van Aelst & Van Went 1991), *Parietaria* (Vega-Maray 2002) and *Zygophyllum fabago* (Castells 2000).

After 10 min of activation the *Plantago lanceolata* pollen grains show a high activity of dictyosomes and a huge production of dictyosomal vesicles. These vesicles may be destined to the formation of the pollen tube wall. A large number of dictyosomal vesicles were detected in pollen tube of *Nicotiana glauca* (Cresti et al. 1992), *Lilium* (Van der Woude et al. 1971; Miki Hirose & Nakamura 1983), *Papaver* (Cresti et al. 1990; Van Aelst & Van Went 1991), in *Zygophyllum* (Castells 2000) and *Prunus* (Arcalís 2001).

The concentric dictyosomes were also observed in *Zygophyllum fabago* (Castells 2000) and *Parietaria judaica* (Vega-Maray 2002). This conformation could be related to the increase in the number of these organelles, since the vesicles secreted by the *trans* face could be merged together and form new cisternae, which then will lead to new Golgi complex.

The presence of gold markers in rough endoplasmic reticulum and in Golgi apparatus increased in activated pollen grains of *Plantago lanceolata*, as occurs in other pollen types (Casas et al. 1996; Alché et al. 2002; Vega-Maray et al. 2006b). This labelling can be explained by the role of these organelles. The rough endoplasmic reticulum produces new proteins, some of them migrate to the Golgi complex. Golgi apparatus have two functions in the vegetable cells: synthesise glycoproteins and polysaccharide complex of the cell walls (Zhang & Staehelin 1992). The transport of particles between both organelles is the cause for which labelling also appears in rough endoplasmic reticulum vesicles and in Golgi stacks of *P. lanceolata* pollen grains. The localisation of Pla 1 1 in both organelles suggests an active process of transport, as described by other authors in other pollen grains (Nebenführ & Staehelin 2001; Suárez-Cervera et al. 2005).

The number of ribosomes into the activated pollen grains increases with respect to the mature pollen grains, in some cases to clump forming polyribosomes. According to Charzynska et al. (1989) and Vega-Maray (2002), polyribosomes are the sites of synthesis that the pollen needs for pollen tube growth.

The vegetative nucleus does not seem to have undergone many changes during activation, but what has been observed is an increase in the number of nuclear pores, as described in *Brassica napus* (Straatman et al. 2000). In other pollen grains no changes were observed in the nucleus of vegetative cells (Casas et al. 1996; Márquez 1996; Castells 2000; Arcalís 2001; Vega-Maray 2002).

Spermatic cells do not appear to change during the activation of *Plantago lanceolata* pollen grains. The lack of change coincides with the observations of Castells (2000) in *Zygophyllum fabago*, Arcalís (2001) in *Prunus dulcis* and Vega-Maray (2002) in *Urticaceae*.

We found gold marked in the nucleus of vegetative cell and in sperm cells too; it indicates that both types of cells are involved in the process of recognising pollen-stigma as noted by Castells et al. (2002) and Vega-Maray et al. (2006a).

Localisation of allergens in pollen grains could help to understand the role of these allergens. Gao et al. (2019) localised four allergens in *Artemisia* pollen, each one with different patterns of localisation. Pollen allergens only belong to a few protein families, such as expansins, profilins, and calcium-binding proteins (Chen et al. 2016).

Ole e 1, the main allergen of olive pollen, exhibits a broad degree of amino acid sequence identity with proteins of other species, like *Plantago lanceolata* (Villalba et al. 2014; Asam et al. 2015). These proteins have been indexed as members of the so-called pollen proteins of the Ole e 1 family and its biological function is not yet known (Castro et al. 2007; Villalba et al. 2014). A study carried out by Stemeseder et al. (2016) showed that the crystal structure of Pla 1 1 has a structural similarity and allergenic divergence within the Ole e 1-like profiling family. The Ole e 1 family therefore share the same core structure, while loop regions can be heterogeneous. Especially, Pla 1 1 lacks the C-terminal region, previously shown to be an important epitope of Ole e 1. It limits antibody cross-reactivity between Pla 1 1 and Ole e 1 family.

The structural similarities and similar localisation between Ole e 1 and Pla 1 1 might account for a shared biological function in the pollen grain (Castro et al. 2007). This author indicated that members of this group of allergens show sequence similarity

with soybean trypsin inhibitor-related proteins, but no conclusive evidence of this putative relationship has been established to date.

However, Songnuan (2013) pointed out that LAT52, a highly expressed protein in the mature pollen of tomato, is similar to Ole e 1. It is likely that these proteins play significant roles in pollen tube guidance.

Conclusions

Our study shows that *Plantago lanceolata* pollen grains experience several modifications during the hydration and activation processes. These modifications affect the cell wall and organelles such as the endoplasmic reticulum and Golgi apparatus. Throughout these processes there is an increase in the production of Pla l 1, the major allergen of *P. lanceolata*. This suggests that Pla l 1 is probably synthesised in the rough endoplasmic reticulum, transformed in Golgi apparatus and transported in dictyosomal vesicles through the cytoplasm of the vegetative cell towards the wall. The vesicles fuse with the plasma membrane releasing the allergen outside. The increase in the production and release of this allergen when pollen grains are activated suggest that Pla l 1 has an important role in pollen-stigma recognition and in the subsequent development of the pollen tube.

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No potential conflict of interest was reported by the authors.

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