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POLLEN WALL AND STORAGE SUBSTANCES IN *ACTINIDIA DELICIOSA* POLLEN

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SUMMARY: In pollen extracts trace amounts of flavonoids and higher concentrations of other phenolics were detected. Callose was located at the pollen tube wall and callose plugs inside the pollen tube. Pectins were located in intine namely at the aperture zone and in the tube wall extension. Proteins were detected in the pollen coat, intine, near the pore region, at the aperture chamber level and also in the cytoplasm in mature and germinated pollen. In pollen, cytoplasmic lipid granules were observed. During germination, lipidic bodies were less numerous. Pollen grain amyloplasts presented numerous starch grains, which disappeared during germination. Results were confirmed at the ultrastructural level using electron microscopy.
KEY WORDS: *A. deliciosa* cv. *Tomuri*, flavonoids, callose, pectins, proteins, lipids.

RESUMEN: En los extractos de polen se detectaron vestigios de flavonoides y concentraciones más altas de otros fenólicos. Se localizaron sustancias calosas en la pared del tubo polínico y tapones calosos dentro del tubo de polen. Se detectaron pectinas en la intina, sobre todo en la zona de abertura y en la extensión de la pared del tubo. Se visualizaron proteinas en intina cerca de la región de los poros y en el citoplasma del polen maduro y germinado. Se observaron gránulos lipídicos citoplásmicos. Durante la germinación los cuerpos lipídicos fueron menos numerosos. Los resultados fueran confirmados por microscopía electrónica.
PALABRAS CLAVE: *A. deliciosa* cv. *Tomuri*, flavonoides, calose, pectinas, proteinas, lípidos.

INTRODUCTION

Actinidia deliciosa, usually known as kiwifruit, is a dioecious species that produces both staminate and pistilate flowers that produce non-functional reproductive structures of the opposite sex. Pollen from male flowers presents all its characteristic features but pollen from female flowers is devoid of content, consisting entirely of an exine wall.

The pollen of many Gymnosperms and Angiosperms show a characteristic and often intense pigmentation. This pigmentation is due to the carotenoids, that are located in the exine, and flavonoids, which are thought to be located in or at the outer pollen wall (Wiermann & Vieth, 1983).

Flavonoids are a major class of low molecular weight secondary metabolites that, in higher plants, are responsible for several functions. Among these functions, flavonoids protect plants from predators and infectious agents, act as UV radiation shields, are signal molecules in plant-bacterium symbiosis, such

as nitrogen fixation, and finally these pigments are responsible for the attraction of pollinators and seed dispersal agents (Burbulis *et al.*, 1996).

One particular class of flavonoids, flavonols, is required for pollen germination and pollen tube growth in maize and petunia (Mo *et al.*, 1992, Deboo *et al*, 1995, Xu *et al.*, 1997). This need is not universal among Angiosperms since *Arabidopsis* plants deficient in flavonoid biosynthesis appear to be fully fertile (Burbulis *et al.*, 1996).

For many species, it has been chemically demonstrated that the main pollen tube wall component is a beta-1,3-glucan: callose. Generally, callose is identified on basis of its staining with aniline blue or its fluorescence induced by the aniline blue fluorochrome. Normally this b-1,3-glucan is present in pollen tube walls (extracellular deposition), in the callose plugs along the pollen tube (intracellular deposition) and is absent in the tip growing region (Cresti & van Went, 1976, Hasegawa *et al.*, 1996).

According to the state of pollen development, callose has different functions. So, during pollen development and initial germination the callose layers subtending pore areas are readily mobilised as reserve substances. In developing pollen, the callose wall act as a molecular sieve to enable the autonomous development of the haploid pollen nuclei, independently segregated to their own cytoplasm. At last, by forming depositions along the inner tube membrane during germination, callose adds wall strength and delimits tube cytoplasm. Callose plugs, formed in the back of the growing pollen tube, limit and contain the path of cytoplasmic stream inside the tube (Stanley & Linskens, 1974).

Pectins, probably the most complex class of polysaccharides, are one of the major component of all cell walls. These substances are known to play a role in the adhesion of adjacent cells. Pectins are synthesised in the Golgi apparatus and they are secreted as highly methylesterified forms that are soon de-esterified by a pectin methylesterase producing unesterified pectins upon arrival in the cell wall. These pectins are transported to the tube tip region and participate in the building of new regions of wall and membrane (Juah & Lord, 1996).

In pollen grains, proteins are present both in the cell wall and in the protoplasm. Peptide fractions found in the pollen coat can also be observed in the protoplasm; however, numerous peptides are characteristic from the pollen coat and others from the protoplasm (Kalinowski *et al.*, 2002).

A single pollen grain contains several thousands of different enzymes and isozymes, but only about 100 non-enzymatic proteins (Stanley & Linskens, 1974). It ha been reported that during the first minutes of germination the total amount of proteins present in the cytoplasm increases (Bagni *et al.*, 1981 and Liu & Ger, 1997).

Proteins from the pollen wall are known to be related with the pollen recognition process, enzymes necessaries for pollen germination and pollen tube elongation, and allergenic proteins. Observation in MALVACEAE as well as in other families revealed that the intine-held proteins of Angiosperm pollen grains are always of gametophytic origin, while those held in exine cavities are from sporophytic origin (Heslop-Harisson *et al.*, 1973).

Calvino (1952) proposed a scale of lipids and starch content to classify more than 1,170

pollen species. She also suggested that bees distribute fat-rich pollens, while starch-rich pollens are transported by wind. Lipid quantification, using pollen ether extracts, indicates values ranging from 1 to 20% dry weight (mean- 5%) (Stanley & Linskens, 1974).

Several roles for the lipids present in pollen grains have been suggested. They can function as storage source of energy required for pollen tube elongation, as growth substances, as membrane components after decomposition and reorganisation and as nutritive source for bees (Stanley & Linskens, 1974 and Noguchi, 1990).

Starch is the only insoluble polysaccharide stored temporally in plastids during pollen development. Although starch quantities are highly variable in pollen, ranging from 22.4% in *Zea mays* to 1.4% in *Lilium auratum*, constitute the major fraction of dry matter in pollen (Stanley & Linskens, 1974 and Bellani *et al.*, 1985). Environmental factors, such as humidity level and temperature, can induce changes in this storage substance content (Stanley & Linskens, 1974).

Amylogenesis and amylolysis are phenomena shared by all the microspores and pollen grains of a loculus, but they do not behave synchronously. In dicotyledons two waves of amylogenesis/amylolysis occur after meiosis and in monocotyledons there are more than two of these waves (Pacini & Franchi, 1988).

MATERIAL AND METHODS

POLLEN COLLECTION

Experiments were performed with pollen collected during the 2001 blossoming season using plants of *A. deliciosa* cv. *Tomuri* from the Northwest of Portugal (Minho Litoral region).

Several male flowers were harvested and brought to the laboratory where the undehisced anthers were removed. Anthers were dehydrated at 27ºC and 35% relative humidity for 12 hours. Pollen was collected from anthers and preserved at -20ºC.

IN VITRO GERMINATION

Samples were germinated for 2, 4 and 6 hours at 30ºC, in a thermo-controlled dryer, at 37ºC, with shaking. The germination medium used in these experiments contained sucrose, boric acid and calcium nitrate (Abreu *et al.*, 1997).

FLAVONOID AND OTHER PHENOLIC COMPOUNDS:

For phenolic compounds extraction, pollen was added to 70% aqueous methanol, shaken, centrifuged at 12.000 g and passed through a 0,45 mm mesh.

For HPLC, Merck Lichro CART 250-4 and LiChrospher 100RP-18 (5 mm) columns were used. The solvents used were methanol (solvent A) and acidic water (1% formic acid) (solvent B). The flow rate was 1mL/min with the following gradient: 20 min - 40%; 25 min - 50%; 40 min - 50%; 45 min - 70%.

HISTOCHEMISTRY

For callose localisation, we used fresh pollen grains and fresh germinated pollen (four hours). The material was stained with 0,2% alcian blue in 0,1N HCl, for one hour, in the dark and observed in a fluorescence microscope under visible and ultraviolet light.

For pectins, we used fresh and germinated pollen. The material was stained with

0,2% aniline blue, for one hour, in the dark. Then, it was observed under visible and UV light.

Histochemical studies were performed in fixed material sections and in mature and germinated pollen without fixation.

Pollen grains and germinated pollen (4 hours) were fixed in 2,5% glutaraldehyde in 0,1M cacodylate buffer at pH 7,2, for three hours and post-fixed in 2% osmium tetroxide in the same buffer, for two hours. After fixation the samples were treated with 1% aqueous uranyl acetate for 30 minutes, dehydrated in a graded acetone serie, and with propylene oxide and embedded in Epon 812.

Concerning detection of proteins, the semithin sections placed in glass slides were submerged into toluidine blue stain (0,1% toluidine blue dissolved in 1% sodium tetraborate), for one minute. Then the glass slides were carefully washed in distillate filtered water.

Histochemical detection of lipids was performed using Sudan black B stain. The semithin sections placed in a microscope slide were submerged into 70% ethanol and then into Sudan black B solution, for one minute. At last, the glass slides were placed in absolute ethanol for one minute.

For starch localisation, we also used semithin sections. The material was stained with 0,5% iodine solution, for two minutes. The glass slides were carefully washed.

To observe insoluble polysaccharides, we used the PAS reagent. The semithin sections placed in a glass slide were submerged into 70% ethanol and then into 4% periodic acid, for 20 minutes, at room temperature. Glass slides were submerged into 70% ethanol and washed twice. The slides were left in Schiff reagent for two hours, at room temperature, in darkness, and washed in water for 10 minutes.

The glass slides stained with toluidine blue, Sudan black B, iodine solution and PAS were observed in a light microscope. All results were confirmed at the ultrastructural level (TEM).

RESULTS

POLLEN GERMINATION

In vitro pollen germination was assessed after 2,4 and 6 hours but no significant differences were observed between 4 and 6 hours. Pollen grains usually germinate 30 minutes after sowing under our experimental conditions and develop long pollen tubes quite rapidly.

FLAVONOIDS AND OTHER PHENOLIC COMPOUNDS

In *A. deliciosa* pollen extracts trace amounts of flavonoids and relatively higher concentrations of other phenolic compounds were detected (Tab. 1and Fig. 1). Due to the lack of convenient standard solutions it was not possible to identify the nature of these compounds but regarding their retention time they probably are derivatives of *p*-coumaric and caffeic acids.

HISTOCHEMISTRY

Callose detection was performed using aniline blue. Pollen tube wall and callose

Total phenolic compounds	Flavonoids
9,3059 ± 0,4928	2,1446 ± 0,0918

TABLE 1. Flavonoids and total phenolic compounds (mg/g pollen) measured by spectrofotometry.

plugs in the pollen tube cytoplasm were blue, under visible light, and fluoresced under ultraviolet light. The pollen tube tip, which is the growth region of the pollen tube, was not stained. (Figs 2a and 2b).

Using alcian blue, pectins presented a blue coloration and were located in intine namely at aperture region (Figs 2c and 2d).

Under light microscopy, with toluidine blue, in mature and germinated pollen, proteins were detected by its characteristic blue colour in the pollen coat, intine, near the pore region, at the aperture chamber level and also in the cytoplasm (Figs 3a and 3b). The exine exhibited a green coloration probably due to the presence of polyphenols.

Using Sudan black B, mature pollen exine and cytoplasmic lipid granules positively stain (Figs 3c). According to the level of germination, lipidic bodies were less numerous (Figs 3d), so in the beginning of germination these bodies were more numerous than in the pollens with well developed pollen tube.

Mature pollen grains presented amyloplasts with numerous starch grains revealed using iodine solution (Fig. 3e). The amount of starch decreased during pollen tube elongation (Fig. 3f).

The results concerning the presence or the absence of lipid and starch in the cytoplasm of mature and germinated pollen were confirmed using transmission electron microscopy. In mature pollen (Fig. 4a), abundant lipidic bodies and plastids filled with starch were present. After germination (Fig. 4b), some lipids were present in the cytoplasm but no starch was observed in the plastids.

In mature pollen grains, insoluble polysaccharides (PAS test) were detected in the cytoplasm, pollen coat, intine and at the apertural chamber level (Fig. 3g). During pollen tube elongation the insoluble polysaccharides are partially consumed, persisting in the cytoplasm (Fig. 3h). So, in mature pollen and during germination, the PAS staining is comparable with the iodine staining.

DISCUSSION

POLLEN GERMINATION

We found different composition of germination medium for *Actinidia* species (González & Coque, 1994, Abreu *et al.*, 1997 and Bomben *et al.*, 1999). After testing them

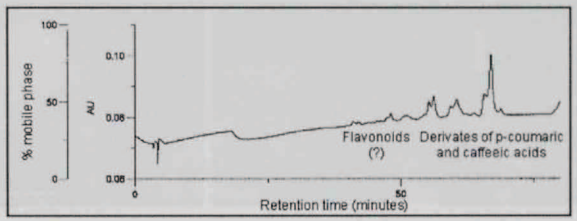

FIGURE 1. HPLC profile of *A. deliciosa* pollen extracts (350 nm).

all, the *in vitro* germination medium showing highest percentage was the one proposed by Abreu *et al.*, (1997).

FLAVONOIDS AND OTHER PHENOLIC COMPOUNDS

In *A. deliciosa* cv. *Tomuri* pollen extracts trace amounts of flavonoids and other phenolic compounds were detected. It has been reported that flavonoids have a role in pollen dissemination, either by attracting pollinators or by acting as protective shields against ultraviolet radiation (Burbulis *et al.*, 1996). Maize and petunia male sterility can be overcomed if the germination medium is supplemented with flavonoids (Mo *et al*, 1992, Deboo *et al.*, 1995 and Xu *et al.*, 1997).

On other hand there are plants, like *Arabidopsis*, in which flavonoids are not essential for pollen germination (Burbulis *et al.*, 1996). *A. deliciosa* pollen germinates perfectly well without the external addition of flavonoids.

It is also known that kiwifruit pollen is mainly transported by wind and honeybees transport only a small part (Testolin *et al.*, 1991) compounds are absent from pollen surface. However, some authors suggest that honeybees and wind are equally important in pollen transfer (Howpage *et al.*, 1998) but Malabouef (1996) refers that honeybees play the major role in kiwifruit pollination.

HISTOCHEMISTRY

Callose was detected in the pollen grain, in the pollen tube wall and forming callose plugs. This staining pattern was already described by Stanley & Linskens, 1974.

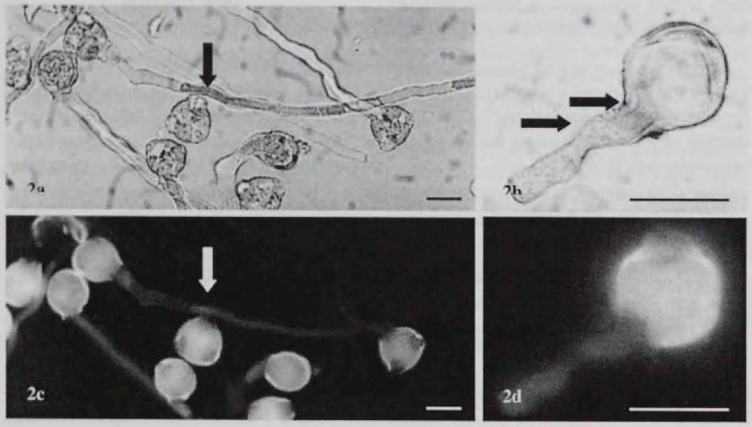

FIGURE 2. Callose detection with aniline blue, under visible (a) and UV-light (b); detection of pectins with alcian blue, under visible (c) and UV-light (d) (bar 20 mm).

Regarding the function of these callose depositions, the extracellular callose could be involved in the binding of water by imbibition, thereby maintaining the osmotic conditions in the pollen tube (Cresti & Van Went, 1976).

Pectins were located in intine namely at aperture zone and along all pollen tube wall extension forming ring-like structures.

In previous works it was recorded that near the germination aperture, both in pollen grains and pollen tube, pectins were located in the intine. It was proposed that these polysaccharides may play an important role in the cell wall formation during pollen tube formation. In the same works, a periodic annular pattern of pectins distribution was observed (Li *et al.*, 1995, Juah & Lord, 1996 and Mogami *et al.*, 1999).

In mature and germinated pollen, proteins were detected in the pollen coat, intine, near the pore region, at the aperture chamber level and also in the cytoplasm. The green coloration present in the exine may probably be due to the accumulation of polyphenols.

Using Sudan black B, mature pollen exine and cytoplasmic lipid granules positively stain. Lipidic bodies were less numerous after pollen germination. In *Tradescantia reflexa* pollen tubes, a newly membranous structure was reported. This membranous structure may be involved in the decomposition of lipidic granules and from which vacuoles might be originated (Noguchi, 1990).

Mature pollen grains presented amyloplasts with various starch grains revealed at optical microscopy using iodine solution and at transmission electron microscopy. The amount of starch decreased during pollen tube elongation.

Insoluble polysaccharides were detected in the cytoplasm, pollen coat, intine and at the apertural chamber level but they were

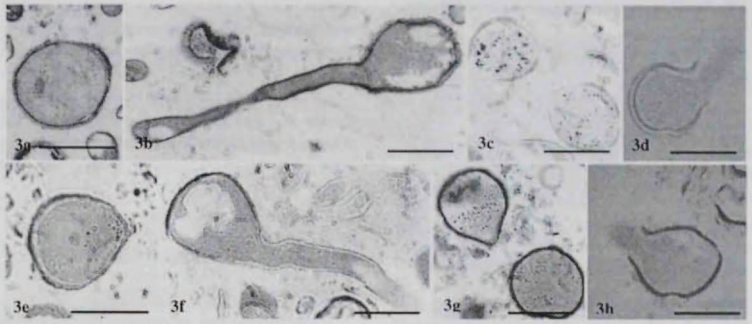

FIGURE 3. Detection of proteins in mature (a) and germinated pollen (b); detection of lipids in mature (c) and germinated pollen (d); detection of starch in mature (e) and germinated pollen (f); detection of polysaccharides in mature (g) and germinated pollen (h) (bar 20 mm).

partially consumed during germination. So, in mature pollen and during germination, the PAS staining is comparable with the iodine staining.

CONCLUSION

Regarding all our results we can conclude that flavonoids and other phenolic compounds are present in very small amounts in these pollen extracts.

Callose was detected at the pollen tube wall and callose plugs inside the pollen tube.

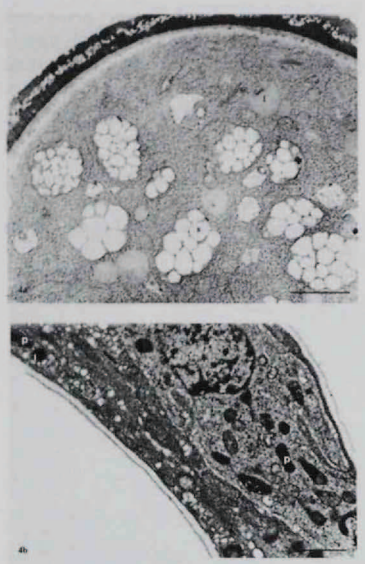

FIGURE 4. TEM micrographs of mature pollen grain (4a) and pollen tube (4b): In the mature pollen, amyloplasts are filled with starch (s) and lipidic bodies (l) are present in the cytoplasm. In the pollen tube a few lipidic bodies are observed and plastids (p) without starch.

Pectins were located in the intine namely at aperture zone and in all pollen tube wall extension arranged in ring-like structures.

In mature and germinated pollen, proteins were present in the pollen coat, intine, near the pore region, at the aperture chamber level and also in the cytoplasm.

The results showed that amylogenesis occurred during early and middle two-celled pollen stage. During germination, lipids and starch were hydrolysed and probably used as energy sources for the rapidly growing pollen tubes. The insoluble polysaccharides were not completely consumed persisting in the cytoplasm.

Further works are being undertaken to investigate their role in the building up of the pollen tube wall.

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