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POLLEN DEVELOPMENT OF *TABEBUIA PULCHERRIMA* SANDWITH (BIGNONIACEAE) FROM MEIOSIS TO ANTHESIS[1]

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Abstract – [Pollen development of *Tabebuia pulcherrima* Sandwith (Bignoniaceae) from meiosis to anthesis]. The pollen ontogeny of *Tabebuia pulcherrima* was analyzed. Pollen development follows the usual course in dicotyledonous angiosperms. The sporoderm is composed of a finely reticulate outer exine, and a thin intine. The microstructure of the exine is determined by the primexine, before the separation of the microspore tetrads by dissolution of the callose wall. At the aperture sites, the pectinized intine layer forms an oncus impregnated with proteinaceous substances. Irregularly shaped ruptures occur in the colpal membrane, partially exposing the intine. During meiosis, the callose walls of the meiocytes gradually become PAS-positive, and a cycle of amylogenesis/amylolysis takes place in the cytoplasm. At tetrad stage the meiotic amyloplasts are almost absent. A new cycle of amylogenesis/amylolysis is observed in the protoplasm of the pollen vegetative cell, during its maturation. At the same time, the vegetative cell nucleolus swells and develops conspicuous nucleolar vacuoles. The generative cell does not develop amyloplasts. In mature pollen grains, the generative cell is elongated and falciform, with a PAS-positive wall, and becomes associated with the vegetative nucleus to form the male germ unit.

Resumo – [Desenvolvimento do pólen de *Tabebuia pulcherrima* Sandwith (Bignoniaceae) da meiose à antese]. A ontogenia do grão de pólen de *Tabebuia pulcherrima* foi analisada. O desenvolvimento do andrófito segue o modo usual em angiospermas dicotiledoneas. A esporoderme é composta por uma exina finamente reticulada e um delgado estrato inferior pectinizado (intina). A microestrutura reticulada da exina é determinada pela primexina, antes do desmembramento das tétrades por dissolução da calose. Nos colpos, o estrato pectinizado torna-se um oncus impregnado por substâncias protéicas. A exina das membranas aperturais sofre rupturas irregulares, expondo parcialmente a intina. No período meiótico as paredes de calose dos meiócitos tornam-se progressivamente PAS-positivas, ao passo que, no citoplasma, uma onda de amilogênese/amilólise é observada, estando os amiloplastos virtualmente ausentes nas tétrades de andrósporos. Durante a maturação do grão de pólen, uma nova onda de amilogênese/amilólise acontece no protoplasma da célula vegetativa, ao passo que, no seu núcleo, o nucléolo expande-se significativamente, desenvolvendo conspícuos vacúolos nucleolares. A célula generativa não desenvolve amiloplastos. No grão de pólen maduro, a célula generativa torna-se alongada, com parede celular PAS-positiva e associa-se ao núcleo da célula vegetativa, formando a unidade germinativa masculina.

Key words: Bignoniaceae, *Tabebuia*, pollen ontogeny, histochemistry.

Introduction

The initial data on the ontogenetic development of the pollen grain in the family Bignoniaceae was provided by Duggar (1899), who investigated the microsporogenesis and some other aspects of pollen development of *Bignonia venusta* (= *Pyrostegia venusta*). Later, studies emphasizing cytological aspects provided data regarding microspore mother-cells (MMC) and meiosis in *Spathodea campanulata* (Raghavan & Venkatasubban 1940), *Crescentia cujete*, *Parmentiera serratifolia* and *Dolichandrone reedii* (Venkatasubban 1945). Ghatak (1956) investigated the formation of the MMC and meiosis in the anther of *Oroxylum indicum*. Gupta and Nanda (1978a, b, 1983) and Nanda and Gupta (1978, 1983) performed studies on anther ontogeny and histochemistry of *Pyrostegia ignea* (= *P. venusta*) and *Tecoma stans*, including aspects of pollen development. The microsporogenesis and other aspects of the pollen development of *Tabebuia rosea*, *Millingtonia hortensis*, *Dolichandrone falcata*, *Heterophragma adenophylum* and *Stereospermum chelonoides* were supplied by Mehra & Kulkarni (1985). Bittencourt Jr. (1996) described the anther development, including microsporogenesis and other aspects of pollen development in *Tabebuia ochracea*. In a study of the ontogeny of the anther of *Tabebuia pulcherrima*, Bittencourt Jr. and Mariath (1997) investigated the development of the MMC, up to pre-meiotic stage. Galati and

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Strittmatter (1999) reported on microsporogenesis and microgametogenesis in *Jacaranda mimosifolia*.

There is a considerable literature on aspects of structural development in the anther of angiosperms. The emphasis of these studies has focussed on the application of histochemical techniques, in order to understand the basic chemical changes that occur during successive stages of anther growth and differentiation. However, there are only a few histochemical studies relating to the development of the anther in Bignoniaceae. The most relevant are those of Gupta and Nanda (1983) in *Pyrostegia*, Nanda and Gupta (1983) in *Tecoma*, and Rudramuniyappa and Mahajan (1991) in *Spathodea campanulata*. The development of the anther parietal layers and sporogenous tissue prior to the formation of the MMC has been described by Bittencourt Jr. and Mariath (1997).

The aims of the present study were to investigate microsporogenesis and structural development of the pollen of *Tabebuia pulcherrima*, to contribute to the histochemical study of the ontogenetic process of the pollen, and to supply further information on the taxonomy of the family.

Material and Methods

The material for the study was collected from August to November of 1993 and 1994. Inflorescences containing floral buds, in a range of developmental stages, were collected from one wild source (ICN 106186), and three cultivated trees (ICN 106184 106185 and 103826) located in the metropolitan area of Porto Alegre, Brazil.

The anthers were removed from the flower buds, and fixed in 2% glutaraldehyde in 0.1M sodium phosphate buffer, pH 6.8 (Gabriel 1982). The fixation was done at room temperature, immediately after each collection (Robards 1985). After fixation the material was stored in 70% ethanol and kept in a refrigerator. After ethyl dehydration, the pieces were infiltrated with hydroxyethylmetacrylate (Historesin-Jung). The blocks were sectioned in a Leitz 1400 microtome, at a thickness ranging from 1 to 4 µm. The sections were mounted on glass slides and subjeckted to histochemical tests, or to staining with Toluidine Blue O (O'Brien & McCully 1981), Acid Fuchsin and Toluidine Blue O (O'Brien & McCully 1981), or Astra Blue and Basic Fuchsin (adapted for historesin sections, starting from Johansen (1940) and Gerlach (1977)). Removal of an eventual excess of Basic Fucsin staining was made by introducing the slides in 50% ethanol. The tendency of the sections to come off slides during Astra Blue, Basic Fuchsin and ethanol treatment was avoided by rapid washing the slides in distilled water and drying on a warming plate at 40°C. This sequence was repeated successive times until the adequate staining of the sections.

Several histochemical tests were performed: the Periodic Acid Schiff reaction (PAS) for the detection of insoluble polysaccharides (except cellulose and callose), after treatment of the sections with hydrazine, an aldehyde blocker (O'Brien & McCully 1981); the IKI test for starch (Sass 1940), conjugated with polarized light; the reaction of Sudan IV (Johansen 1940) and Sudan Black B (O'Brien & McCully 1981) for detection of apolar substances (insoluble lipids); Coomassie Blue (Southworth 1973) as a reagent for total proteins; and Aniline Blue for callose, using the fluorochromatic method (Eschrich & Currier 1964). Sections of pollen grains were also analyzed for the presence of acid polysaccharides, including pectic acids using the Ruthenium Red test (Southworth 1973).

Photomicrographs were taken using a Leitz Dialux 20 EB microscope, and Kodak T-MAX ASA 100 negative film. Drawings were made using the same microscope with a drawing device (camera-lucida).

The pollen grains were also examined with scanning electron microscopy (SEM). The undehised anthers were dehydrated and submitted to critical point drying (Gersterberger & Leins 1978), using a Balzers CPD 030. The anthers were then mounted on aluminum stubs, and opened to expose the pollen. The samples were coated with gold in a Balzers SCD 050 sputtering system, and examined with a Jeol Series 300 SEM, at 25-30 kV.

Results

Microspore mother cells and meiosis

Callose wall deposition, between the plasmalemma and the cell wall of the microspore mother cell MMC (Figures 1a, b), begins when the flower bud is little more than 3 mm long. Shortly before meiosis, numerous nonpolarizing amyloplasts develop in the cytoplasm of the MMC (Figures 1b, c, 4a, b). The MMC amyloplasts are smaller than those of the middle layer and placentoid tissue of the sporangium (Figure 1b). In the IKI-test, the MMC amyloplasts show a slight reaction only, acquiring a brown color, but they are strongly PAS-positive.

At the beginning of meiosis the MMC attains its maximum volume and acquires a more or less spherical shape, while the callose walls develop to their maximum thickness (Figures 1d, 4b). In the same anther the microsporangia can be at different stages of meiosis, although there is a notable synchronism among the meiocytes within the same sporangium. Although not all the stages of meiosis have been examined, a steady reduction was observed in the amount of MMC amyloplasts until, by the tetrad stage, a total absence

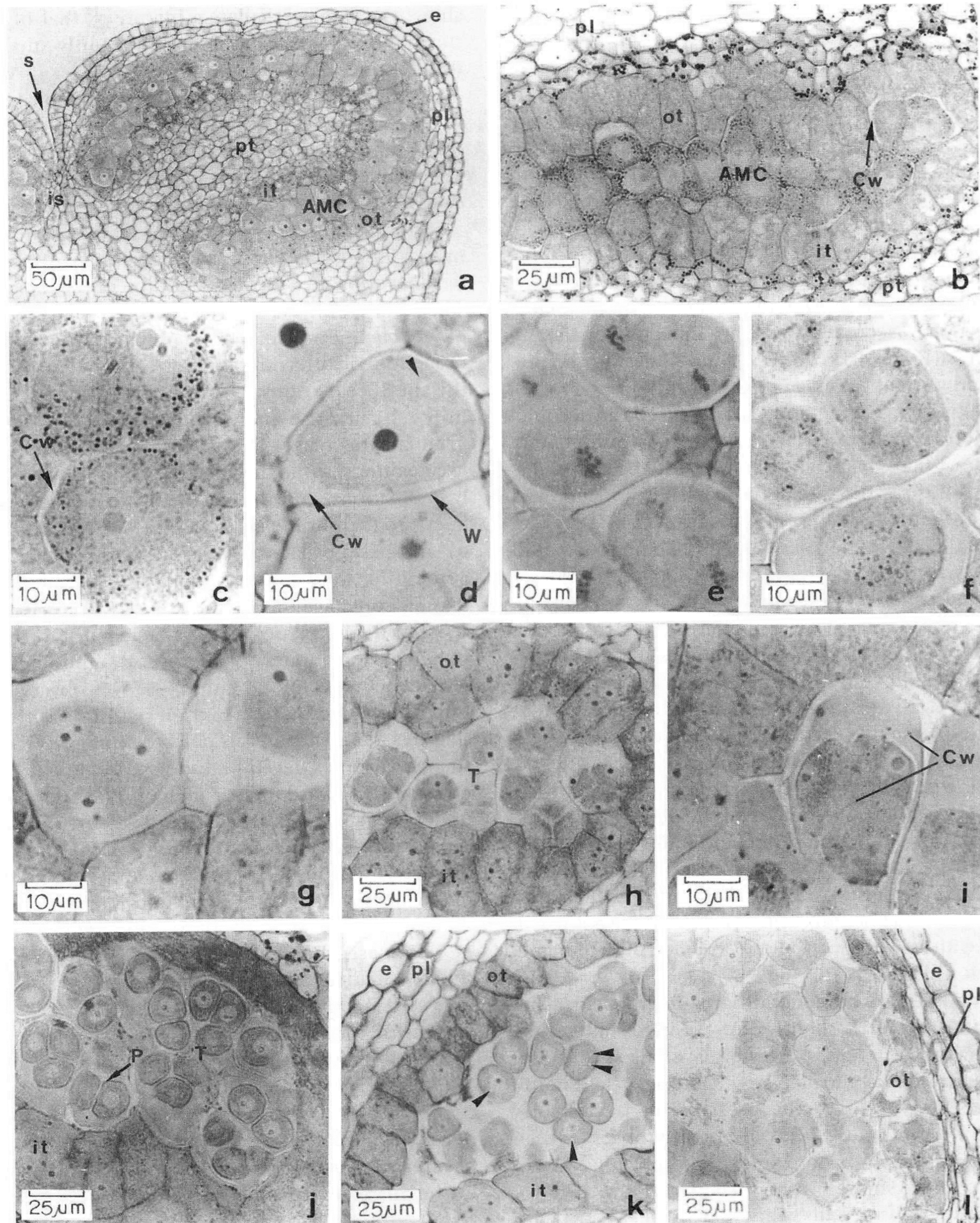

Fig. 1: Transverse sections of anthers in successive developmental stages. (a.) General aspect of microsporangium just before the beginning of the meiosis. (b.) One of the microsporangium corners showing the amyloplasts (black intracellular bodies). (c.) MMC in pre-meiotic stage containing amyloplasts. (d.) Meiocytes at the beginning of Prophase I. The arrow head indicates a conspicuous spherical organelle (possibly a lipidic body). (e and f.) Meiocytes during Metaphase II. (g.) Meiocytes during telophase II. (h and i.) Microspore tetrads immediately after the cytokinesis. (j.) Microspore tetrads after formation of primexine. (k.) Early free-microspore stage. The arrow heads indicate the same structure mentioned in (d). (l.) Microspores during the volumetric expansion after the disruption of the callose walls. Stains: a, d, e, g, h, and k – Acid Fuchsin/Toluidine Blue; b, c, f, i, j, and l – PAS/Sudan Black B. AMC – microspore mother cell; Cw – callose wall; e – epidermis; is – intersporangial septum; it – inner tapetal layer; ot – outer tapetal layer; pl – anther parietal layers; pt – placentoid tissue; s - stomial groove; T – microspore tetrads; W – MMC cellulose wall.

was observed (Figures 1f, i, 4c-f). During Prophase I the callose wall is slightly PAS-positive; however this reactivity increases, reaching a maximum at the end of meiosis. The callose also stains lightly with Astra Blue.

One or two conspicuous spherical organelles were observed close to the meiocyte nucleus, before the end of Prophase I (Figure 4a, b). They stain lightly with Sudan Black B and with Acid- or Basic Fuchsin. These organelles are envelopped in a thin halo that is more translucent than the surrounding cytoplasm. Nevertheless, the halo stains heavily with Coomassie Blue. Such organelles are also present in the other meiotic phases observed, and seemingly, they are inherited by the microspores (Figures 1k, 2b, 4d-l), and persist in the pollen grain until anthesis (Figures 2g, 4m-q).

Cytokinesis is simultaneous and results in tetrahedral microspore tetrads (Figures 1h-j, 4d-f). Subsequently, the original pectocellulosic walls of the MMC dissolve. Callose is degraded and each tetrad separates into four young pollen grains (Figures 1k-l, 4f-g). The pollen sacs fill with locular fluid and begin to expand.

Development of the pollen grains

After tetrad formation, the microspores frequently develop conspicuous nucleolar vacuoles (Figures 4e, f). Before the callose walls disintegrate, and expose the microspores to secretions originating from the tapetum, the primexine differentiates (Figure 1j). In sections stained with Astra Blue and Basic Fuchsin, the primexine is readily identified by the blue color, which contrasts with the pink-staining of the dense cytoplasm. With Toluidine Blue the primexine stains only slightly bluish (this coloration was observed in rehydrated sections), while with Sudan Black B the primexine stains grayish. At the same stage, the sites where the colpi will form are already defined by the absence of primexine deposition (Figure 4f).

A second, and thinner wall layer forms below the primexine. It is just slightly PAS-positive, but stains rapidly with Ruthenium Red. This layer also stains red with Toluidine Blue metachromic staining. These staining properties indicate the presence of pectic substances. Discontinuities were frequently observed between this layer and the primexine, especially where the microspore shows signs of degeneration.

When the callose walls begin to dissolve, a reticulate exine starts to develop on the outher face of the microspore wall. Columellae become apparent, gradually stainig with Sudan Black B, or green with Toluidine Blue metachromic stainig. However, during this period the thickness of the exine is still about equal to that of the primexine prior to the dissolution of the callose envelope (Figure 4g). In tangential sections of recently released microspores (not shown) the exine already shows the same reticulate structure as that of the mature pollen exine, although with slightly smaller cavities (lumina) and thinner muri.

As the exine thickens, and loss of cytoplasmic density occurs, the microspores expand remarkably fast, once exposed to the locular fluid (Figures 1l, 4g-i). Meanwhile, the pectinized intine layer becomes significantly thinner, and is difficult to detect chemically.

After an initial period of rapid increase in volume, microspore exine deposition decreases, but is still detectable even after the degeneration of the tapetal cells. The exine now has two clearly differentiated layers: an outer ectexine and an inner endexine (according to the morphological terminology of Faegri 1956). Towards the aperture margins the height of the columellae (Figures 4i-q), and the diameter of the lumina (Figure 3c), gradually decrease. The mesocolpia are predominantly ectexinous, while the aperture membranes are mainly endexinous, and thinner than the mesocolpia. The aperture membrane lacks columellae (Figures 2a-p, 4i-q), and has a psilate outer surface. Frequently, the degenerating microspores completely lose their protoplasmic content, although the exine continues to thicken.

After the initial period of rapid expansion, although pollen grain volume in a microsporangium is variable, a tendency to volumetric decrease was observed. This is accompanied by the colpi inflection into the grain (Figures 4i-k). During this period, numerous irregular-shaped vacuoles develop in the cytoplasm and the nucleus moves toward the periphery of the grain, where it undergoes karyokinesis (Figures 2a-d, 4k-l). There is no synchronism in microspore mitosis in the sporangium (Figure 2c). A degree of ontogenetic disyncronism remains within the sporangium and may even increase during subsequent stages of development. At the end of Telophase, the nucleus of the generative cell is just slightly smaller than the nucleus of the vegetative cell (Figure 2d), but the former soon decreases in volume. After cytokinesis, the generative cell, smaller and lens-shaped, remains adjacent to the sporoderm, underlying one of the mesocolpia (Figures 2 e-g; 4m). The cytoplasm of both cells once more begins to increase in density. The nucleus of the vegetative cell once again occupies a more or less central position and its nucleolus begins to expand and develop nucleolar vacuoles (Figure 4m).

Amyloplasts, initially very small and scattered, begin to differentiate in the cytoplasm of the male gametophyte while the nucleus is still undergoing mitosis (Figure 4l). In the subsequent phases, only in the cytoplasm of the vegetative cell does the amount and volume of the amyloplasts increase (Figures 2e-m; 4l-o). The cellular wall that separates the vegetative cell and generative cell becomes PAS-positive and the cyto-

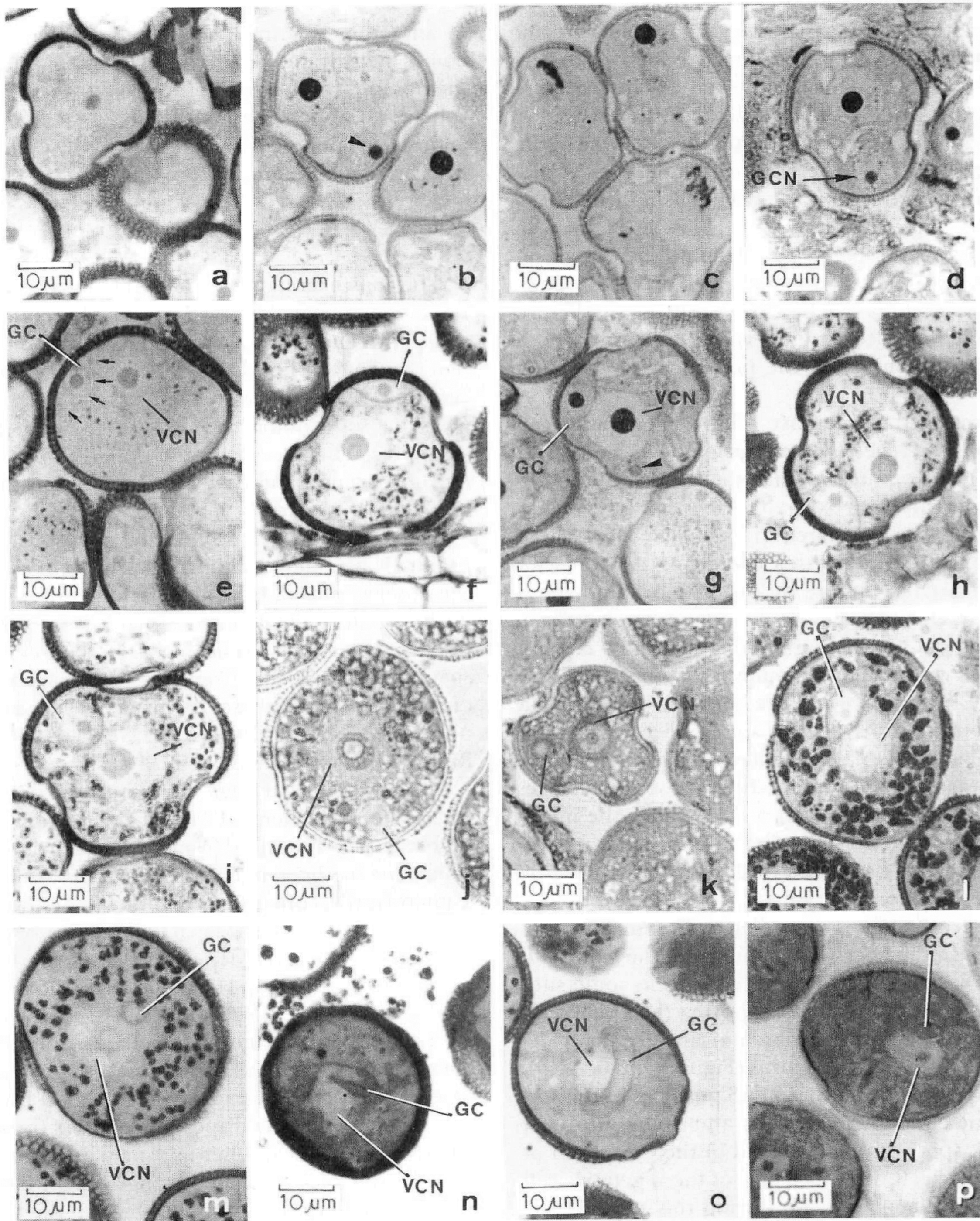

Fig. 2: Pollen grain sections in successive developmental stages. (a.) Microspore at the end of exine thickening stage. (b.) Microspore in mitotic Prophase showing vacuolated cytoplasm. The arrowhead indicates a conspicuous spherical cytoplasmic organelle. Note the chromatin condensations dispersed in the karyolymph. (c.) Three microspores at the same sporangium section but in different mitotic stages (clockwise: Metaphase, Prophase, and the beginning of the Anaphase). (d.) Pollen grain at the end of Telophase. (e.) Pollen grain during cytokinesis. The arrows indicate cell wall formation. (f.) Pollen grain with lens-shaped generative cell adjacent to the sporoderm. (g.) The same as (f), but with different staining. Note the dense cytoplasm of the generative cell. The arrowhead indicates the same structure mentioned in (b). (h.) Pollen grain with the bell-shaped generative cell. (i-k.) Pollen grains seen with three different staining methods, all with a spherical generative cell, totally included in the vegetative cell cytoplasm. Note the nucleolus containing a conspicuous nucleolar vacuole, surrounded by smaller ones. (l.) Pollen grain with a fusiform generative cell. (m.) Almost mature polen grain with falciform generative cell in transverse section. (n and o.) Mature polen grain with the male germ unit viewed at different angles. (p.) Mature polen grain showing dark spots in the vegetative cell cytoplasm. Stains: a, e, f, h, i, l, m, n, and o – PAS/Sudan Black B; b, c, d ,g, and p – Acid Fuchsin/Toluidine Blue; j – IKI test; k – Commassie Blue. GC – generative cell; GCN – generative cell nucleus; VCN – vegetative cell nucleus.

plasmic density of the generative cell becomes greater than that of the vegetative cell (Figures 2g, 4m). The pectinized intine begins to thicken in the aperture areas (Figure 4m), becoming strongly PAS-positive.

Small nucleolar vacuoles can also be observed in the generative cell. The generative cell undergoes expansion, and is projected into the cytoplasm of the vegetative cell, where it assumes a bell shape (Figures 2h and 4n). Soon after being included into the cytoplasm of the vegetative cell, the generative cell becomes spherical and totally contained by a PAS-positive wall (Figure 2i). A gradual reduction of the vacuoles occurs in both cells and the volume of the pollen grain again increases.

Once inside the vegetative cell, the generative cell approaches the vegetative cell nucleus. The generative cell now takes on an irregular shape and rapidly becomes fusiform (Figures 2l, 4o). Amylogenesis reaches a maximum with the starch grains occupying a large proportion of the cytoplasmic content of the vegetative cell. The starch grains are IKI-negative or faintly positive (Figure 2l) and non-polarizing. They often congregate as compound starch grains (Figure 2m). The nucleolus also reaches its maximum size at this stage, and within the nucleolus it is quite common to observe a conspicuous central vacuole, surrounded by smaller vacuoles (Figure 2j). The content of these vacuoles stains with Coomassie Blue (Figure 2k). At the same stage, the intine layer also thickens a little under the mesocolpia (Figures 4o-p), nevertheless it is much thicker under the apertural membrane. In this area it stains intensely with Coomassie Blue.

The pollen grain is now swollen and presents prominent colpi. In the apertural membranes the exine suffers irregular ruptures (Figure 3c), and in some sites may come away in fragments, exposing the underlying intine (Figures 4o-q). The starch grains gradually decrease in volume and quantity (Figures 2m-o, 4o-q), while the cytoplasm becomes PAS-positive. In the sections stained with Acid Fuchsin and Toluidine Blue, small dark spots can be observed in the cytoplasm of the vegetative cell. The nucleolus of the vegetative cell decreases in diameter again during this period (Figures 2p, 4q).

The generative cell does not develop amyloplasts. It becomes elongated, falciform, often with the extremities partially wrapping-around the nucleus of the vegetative cell (Figures 2m-o, 4q). However, in some sections of mature pollen, the generative cell appears to be slightly separate from the vegetative cell nucleus. Its cell wall stays PAS-positive and stains purple with Toluidine Blue. Due to the contrasting nature of its wall, the generative cell is visible even in fixed pollen extracted from dehiscent anthers, without subjecting the pollen to any other treatment. Finally, the pollen grain becomes dehydrated. The colpi close, and the coupus membranes infold, giving the grain (*in vivo*) a prolate spheroidal appearence during anther dehiscence. At anthesis the pollen of *T. pulcherrima* is bicellular, with a finely reticulate exine surface (Figures 3a, b).

Discussion

The conspicuous spherical organelles that appear in the cytoplasm of the MMC and in the pollen grains stains with Fuchsin and with Sudan Black B, suggesting that they may be lipidic in origin. They show a translucent halo that stains with Coomassie Blue, which suggests that these bodies are enclosed in a proteinaceous substance. They persist in the pollen grains until anther dehiscence, and have also observed in the pollen of *T. ochracea* (unpublished data).

In *Tabebuia pulcherrima*, during meiosis, the callose gradually becomes PAS-positive. This is in contrary to the observations of Heslop-Harrison (1964). The PAS-positive nature of the callose that encloses the meiocytes in the anthers has been observed in several other species of angiosperms (Panchaksharappa & Rudramuniyappa 1974, Bhandari & Sharma 1983, Rudramuniyappa & Annigeri 1985, Noher de Halac *et al.* 1992, Rudramuniyappa & Mahajan 1991).

During the tetrad stage, the primexine develops between the plasmalemma of each microspore and the callose wall. Synthesized by microspore protoplast, the primexine has been interpreted as a matrix of cellulosic microfibrils, containing receptors impregnated with protosporopollenin, on which the polymer of the exine wall (sporopollenin) is deposited (Dickinson & Heslop-Harrison 1968, Heslop-Harrison 1963, 1968a, b, 1971, Heslop-Harrison & Dickinson 1969, Knox 1984). When the exine is of baculate (columellate) type, including the cases where the bacula are fused to form crests or muri in a reticulated pattern (Figure 3b), the primexine presents radially disposed receptors (probacula), separated from each other by a matrix substance, but united at the base by a supporting layer with the same chemical constitution of the probacula, the future nexine-1 (foot layer) (Heslop-Harrison 1963, 1968a, b, Dickinson & Heslop-Harrison 1968, Sheldon & Dickinson 1983). However, the cellulosic nature of the primexine could not be demonstrated in the present study, because the observed primexine staining properties with Astra Blue and Toluidine Blue indicate only the presence of acid pectins and polyanionic acid groups. With the degradation of the callose the microspores are released, and come into contact with the tapetally-derived sporopollenin. This triggers the second stage of exine formation (Stanley & Linskens 1974).

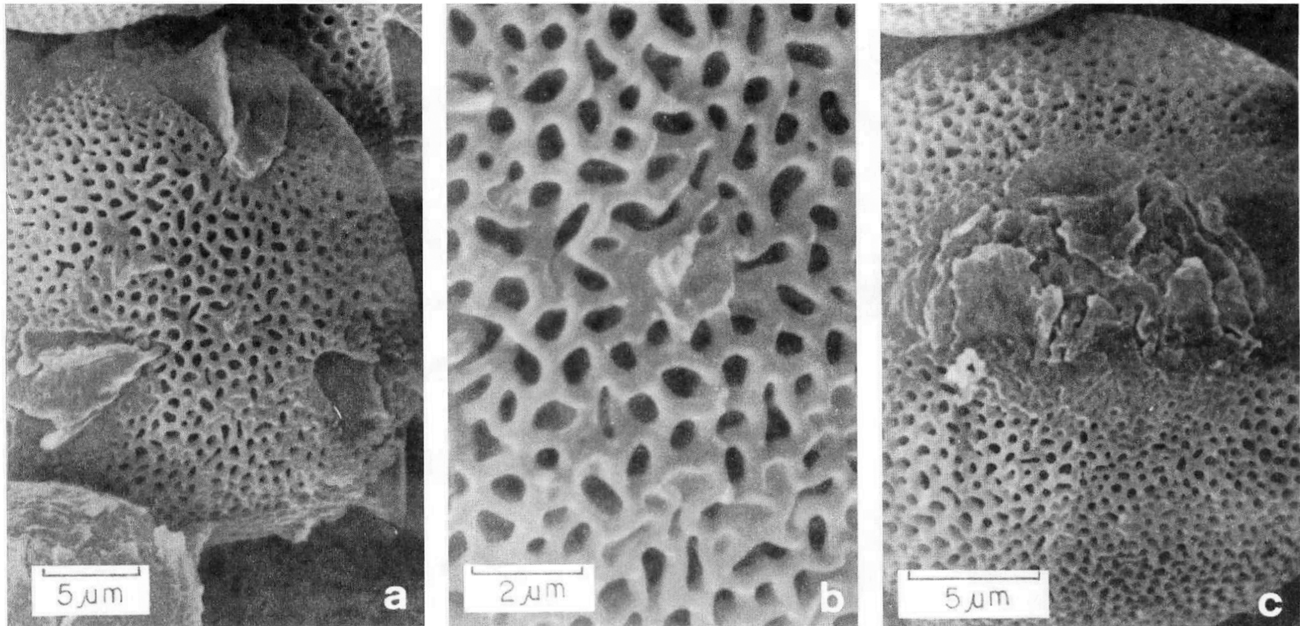

Fig. 3: Scanning electron micrographs of pollen grain. (a.) Polar view. (b.) Detail of the polar area at higher magnification, showing the muri and the lumina of the reticulate exine. (c.) Lateral view showing the apertural membrane.

Although some studies indicated the cellulosic nature of the primexine matrix (Heslop-Harrison 1963, 1968b, Vazart 1970, Rowley 1973), Rowley and Dahl (1977) interpreted the matrix on which the sporopollenin is deposited as being composed of mucopolysaccharide molecules or glycoproteins, intimately linked to the plasma membrane of the microspore, and thereby constituting a glycocalyx. Rowley & Dahl (1977) and Rowley *et al.* (1981a, b) no longer accepted the ectexine as an inert wall, but as a complex cellular material, that can be involved in such processes as ionic changes and recognition mechanisms. In the pollen of *Tabebuia pulcherrima* the primexine is a translucent and homogenous wall layer, strongly staining with Astra Blue, but only faintly with Toluidine Blue or Sudan Black B. Changes in the primexine chemical reactivity was observed immediately after the rupture of the callose wall, because the columellae become visible gradually acquiring the same staining patterns of the mature exine. Similar changes to sporoderm chemical reactivity after callose disruption have been reported for other species (Heslop-Harrison 1968a, b, Rowley & Dahl 1977). At this stage of primexine development, the microspore already show a reticulate wall structure similar to the exine of the mature polen grain, although the muri are slightly thinner. This suggests that the microstructural pattern of the pollen exine of *Tabebuia pulcherrima* was pre-determined during primexine deposition, and only subsequent polymerization of sporopollenin occurs at pre-established sites. The exine microstructural pattern pre-determination by the primexine has been suggested in studies of several other angiosperm species (El-Ghazaly 1999).

The changes in the primexine chemical reactivity cited above was observed at the same time as the microspore expands. The increase in volume also involves a substantial increase in surface area which, consequently, involves a considerable narrowing of the material present in the primexine. However, and this seems to be the case of *Tabebuia pulcherrima*, in many species the narrowing is not observed. Such a phenomenon has been interpreted as the result of rapid sporopollenin deposition on the developing pollen wall (Heslop-Harrison 1968a, 1971).

A fine pectinized wall layer was observed below the primexine. Although this layer undergoes a marked narrowing during the expansion of the free microspore, it thickens again after microspore mitosis, especially in the colpal regions. Due to its position, structure and staining properties in marked contrast to the exine, the pectinized layer has to be interpreted as the intine. However, in some other species intine deposition was only observed following dissolution of the tetrad (Heslop-Harrison 1971, 1975a, Polowick & Sawhney 1993a, Pérez-Muñoz *et al.* 1993, El-Ghazaly 1999). Moreover, the intine was already interpreted as consisting of two distinct layers (Sitte 1953, Bailey 1960), or its outer layer was considered an independent stratum between the exine and the 'true' intine (Ehrlich 1958, Saad 1963). In subsequent studies, Heslop-Harrison (1975a, b, 1987)

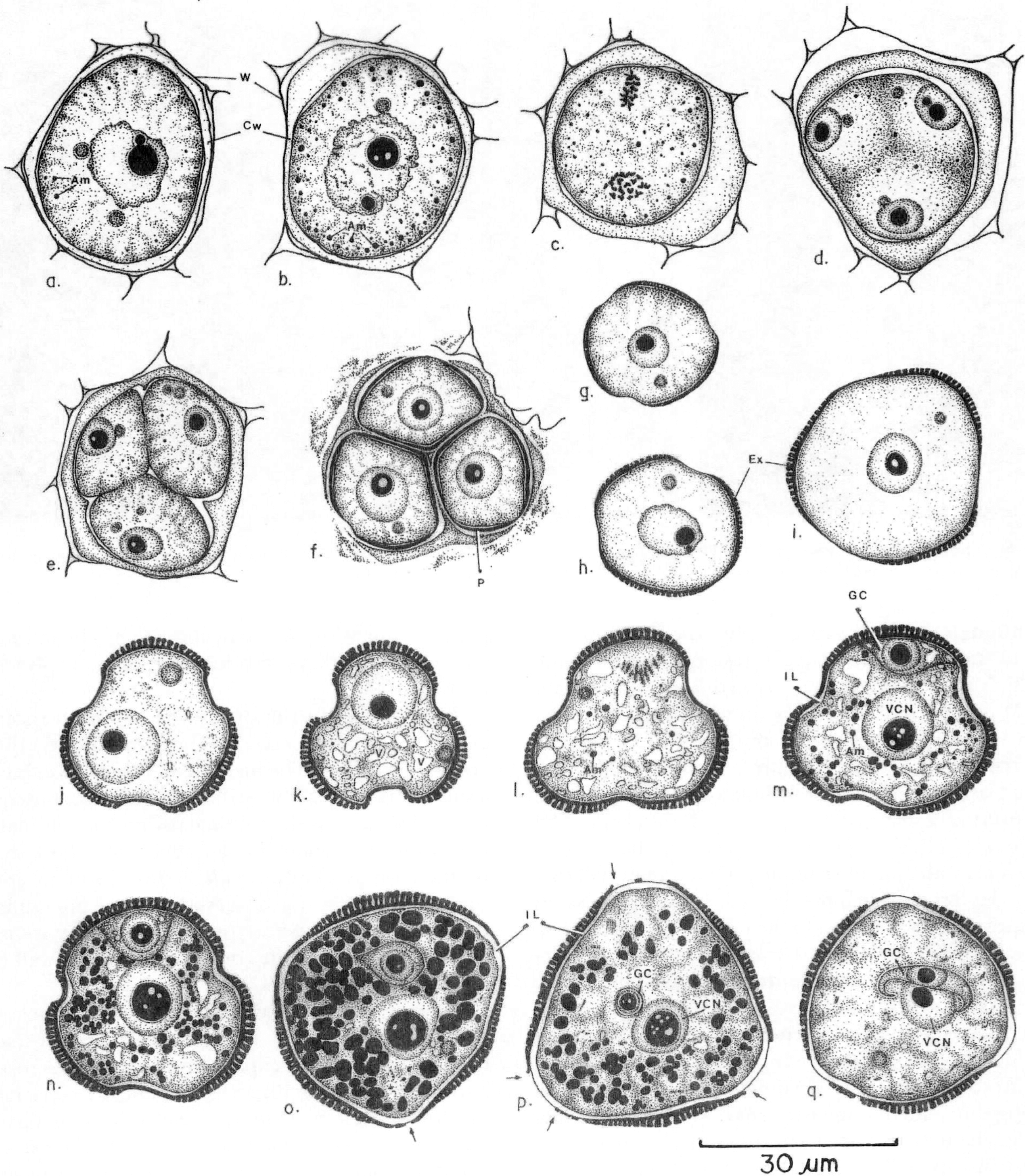

Fig. 4: Drawings summarizing the ontogeny of the pollen grain. (a.) MMC at the pre-meiotic stage. (b.) Meiocyte in Prophase I. (c.) Meiocyte in Metaphase II. (d.) End of the Telophase II. (e.) Cytokinesis. (f.) Microspore tetrad after formation of the primexine. (g- i.) Successive steps during the free microspore expansion. (j.) Microspore with thick exine. (k.) Cytoplasm vacuolation. (l.) Mitosis and beginning of the amylogenesis. (m.) Bicellular pollen grain with lens-shaped generative cell. (n.) Pollen with bell-shaped generative cell. (o.) Pollen with generative cell totally included by the cytoplasm of the vegetative cell. Climax of the amylogenesis and nucleolar expansion in the vegetative cell. (p.) Pollen a little before anthesis. The male germ unit is shown in transverse section. (q.) Amylolysis and the reduction of nucleolar volume in the vegetative cell. The male germ unit is shown in longitudinal section. In (o) and (p) the arrows indicate ruptures of the apertural membrane. Am – amyloplasts; GC – generative cell; IL – sporoderm inner layer (intine); Ex – exine; VCN – vegetative cell nucleus; Cw – callose wall; W – cell wall (pectocellulosic); P – primexine; V – vacuoles.

and Heslop-Harrison and Heslop-Harrison (1991) concluded that the stratification of the sporoderm below the exine is very variable presenting several layers, and that pectins may be present in more then one of these layers. It is important to point out that, according to Saad (1963) the pectic layer underlying the exine may separate from the intine due to the impact involved in the sectioning process. In the preparation work for the present paper, similar discontinuities were observed, but only between the pectinized stratum and the primexine/exine. Furthermore, although the thickness of the sporoderm of *Tabebuia pulcherrima* is much reduced, rendering it impossible to provide a more detailed characterization of its stratification at the optical microscope level, no other wall layer was found below the pectinized layer.

In the apertural areas, the sporoderm parietal layer(s) below the exine usually is swollen to form a lens-shaped, pectin-rich and hygroscopic structure, similar to that of Rowley (1964) called "Zwischenkörper" in pollen of *Poa annua*. However, Rodrígues-García & Fernández (1988) pointed out the equivocal use of the terms employed for the swelling of the apertural membranes in the descriptions of two apparently identical structures, which actually differ in moment, and place of origin. According to Rodrígues-García & Fernández (1988), the lens-shaped structure of the apertures could result from the thickening of either the exine or the intine (or both), each of which occurring at different stages of pollen development. They prefered the use of the term "oncus" (Hyde 1955) to refer to the thickening of the sporoderm apertural regions. In *Tabebuia pulcherrima*, at amylogenesis and afterwards, both the continuity of the thickened inner layer of the apertural membranes with the pectinized layer underluing the mesocolpia, as well as the absence of any layer below, suggest that these swellings in the apertural regions correspond to an intinous oncus (see discussion in Rodrígues-García & Fernández 1988).

The intense staining of the intine, in the apertural areas, with Coomassie Blue confirms the presence of proteinaceous substances. Protein deposition in the inner layer of the apertural membranes has been found in several species. They are reported to be enzymes synthesized by the cytoplasm of the male gametophyte, released later during the germination of pollen grains, and acting on the stigmatic surface, thus allowing the penetration of the pollen tube (Heslop-Harrison 1975a, b, Knox 1984). They may also be gametophytic proteins that participate in pollen-stigma recognition and interaction (El-Ghazaly 1999).

Waves of starch grain synthesis and degradation have been observed in the microspores of many angiosperms (for example, Baker & Baker 1979, Noher de Halac *et al.* 1992, Pacini & Franchi 1988, Franchi & Pacini 1988, Pacini *et al.* 1992, Polowick & Sawhney 1993b). Based on observations of pollen development in *Parietaria judaica*, *Prunus avicum*, *Olea europaea*, *Lycopersicon peruvianum* and *Smilax aspera*, Pacini & Franchi (1988) postulated that, in dicotyledons, two amylogenesis/amylolysis cycles take place in pollen grains, the first being soon after meiosis and the second during the bicellular stage. However, in some angiosperms, including *Pyrostegia venusta* and *Tecoma stans* (Gupta & Nanda 1983, Nanda & Gupta 1983) and *Spathodea campanulata* (Rudramuniyappa & Mahajan 1991), the presence of amyloplasts has been reported at all stages of pollen development. In *Tabebuia pulcherrima*, between the early microspore stage and pollen maturity, only one amylogenesis/amylolysis cycle was observed. The MMC starch grains are hydrolyzed during meiosis, but are virtually absent in the tetrads of recently formed microspores. Pollen grain amylogenesis in *T. pulcherrima* starts during mitosis, reaching a maximum peak soon after the generative cell is immersed in the cytoplasm of the vegetative cell, and recedes afterwards, until the virtual absence of amyloplasts in the pollen of the dehiscent anther.

Starch hydrolysis during pollen maturation has been associated with the deposition of intine and, also with the pollen germination (Pacini & Franchi 1988). In *Tabebuia pulcherrima* the wall layers of the sporoderm are already fully developed during the starch hydrolysis cycle. The emergence and the dissolution of starch grains does not happen simultaneously among pollen grains within the same microsporangium. A similar situation was observed in *Lolium perenne* (Pacini *et al.* 1992) and in *Citrus* (Franchi & Pacini 1988). While the pollen amyloplasts are hydrolyzed, the cytoplasm of the vegetative cell becomes PAS-positive. This is associated with the polysaccharides not being immediately consumed, but remaining dispersed in the cytoplasm as short-chained polysaccharides (Pacini & Viegi 1995). Some authors have suggesting that cytoplasmic polysaccharides in the mature pollen grain are involved in resistance to dehydration and improved pollen longevity (Pacini & Viegi 1995, Franchi *et al.* 1996, Pacini 1996, 2000). The volumetric increase of the pollen grain during the male gametophyte amylogenesis/amylolysis cycle in *Tabebuia pulcherrima* seems to be due to a combination of both the accumulation of starch into the pollen grain, as well as an osmotic effect related to the metabolism of the starch.

Unlike our observations in the vegetative cell, the generative cell of *T. pulcherrima* does not contain amyloplasts. The absence of amyloplasts in the generative cell is common in various angiosperms (Pacini & Franchi 1988, Noher de Halac *et al.* 1992). In *Gasteria verrucosa*, the microspore amyloplasts are polarized during mito-

sis, and totally excluded from the generative cell (Schröder 1985). Degeneration of the generative cell plastids has also been reported (Clauhs & Grun 1977, Vaughn *et al.* 1980). Mogensen and Rusche (1985) attributed the elimination of the plastids to an exocytosis process, during the maturation of the generative cell in barley. Such mechanisms of plastid exclusion of the generative cell are associated with the maternal inheritance of cytoplasmic organelles. Recent studies in *Liriodendron tulipifera* (unpublished results of Mariani, mentioned by Pacini *et al.* 1992) and in *Lolium perenne* (Pacini *et al.* 1992) demonstrate the differentiation of generative cell plastids in amyloplasts, and their subsequent degradation. In the species investigated by Pacini *et al.* (1992), the amyloplasts of the generative cell are apparently destroyed by autophagic vacuoles, and the authors propose such phenomenon as a new control mechanism of the cytoplasmic content of future sperm cells. Polowick & Sawhney (1993a, b) did not find plastids in the generative cell of the pollen of *Lycopersicon esculentum*, but they have found autophagic vacuoles.

During pollen development, significant growth of the generative cell nucleus and nucleolus was observed, accompanied by the formation of conspicuous nucleolar vacuoles. The same phenomenon was observed in sporogenic cells, during the pre-meiotic period (Bittencourt Jr. & Mariath 1997). Nucleolar vacuoles are frequent and conspicuous in recently formed microspores. They can also appear in the generative cell, or even in somatic cells, especially in the meristems, but in a less significantly way. It is accepted that nucleolar vacuoles are related to high biosynthetic activity within the cells that possess them, playing a part in the transport and in the reserves of pre-ribosomic precursors (Moreno Días de la Espina *et al.* 1980). With regard to this aspect it is important to point out the positive result obtained from pollen sections stained with Coomassie Blue. In the meiocytes and in the tetrads, the high synthesizing activity is obviously linked to the meiotic process, and to the beginning of microspore development. In the pollen vegetative cell the expansion and subsequent regression of the nucleolar volume, and of the nucleolar vacuoles, is notably paralleled to the amylogenesis/amylolysis cycle, witch suggests a relationship between the two phenomena. Nucleolar vacuoles were also observed in the vegetative cell of the pollen of *Tabebuia ochracea* (Bittencourt Jr. 1996) and in the immature pollen grains of *Ilex paraguariensis* (Santos & Mariath 1999), during a second amylogenesis cycle.

Mitra (1968) observed ruptures in the apertural membranes of the pollen of *Tabebuia chrysantha* and *Tecomaria capensis*. According to this author, the shape of such openings is not constant, even at the intraspecific level. According to Buurman (1977), a detailed inspection of the pollen apertural membranes of several genera of Bignoniaceae, including *Tabebuia*, reveals the presence of one or more irregular-shaped openings, often without regular positioning. Buurman (1977) also emphasizes that, in acetolysed pollen, such openings appear as holes, but *in vivo* are probably covered by the intine. Leite *et al.* (1982) apparently refer to the same irregular openings since they mention the occurrence, in some species of *Tabebuia*, of "a more fragile area in the colpi in place of the "os", usually of irregular conformation". Bove (1993) equally mentions the existence of irregular ruptures in the colpi of *Cybistax antisyphilitica* and of several species of *Tabebuia*, including the species currently studied. Polowick & Sawhney (1993b) also demonstrated the existence of discontinuities in the colpus exine of mature pollen of *Lycopersicon esculentum*, however, they do not discuss the extent of such discontinuities. Based on the present scanning electromicrographs and sections of the pollen of *Tabebuia pulcherrima*, it is concluded that the pollen begins to present cracks in the thin exine of the colpus membrane and could loosen fragments and originate irregular openings, thus exposing the intine. Therefore, the present observations confirm the interpretations of Buurman (1977).

Although Buurman (1977) speculated as to whether such discontinuities in the exine of the colpi could be a preparation artifact she, nevertheless, proposed that they actually constitute a different apertural system, arising during the ontogeny of the pollen grain, and acting as a sophisticated pre-perforation of the apertural membranes that would facilitate pollen germination. According to Buurman (1977) the rupture of the apertural membranes probably results from unequal resistance of the different exine layers against to tensions imposed during pollen grain development.

The present observations support the hypothesis proposed by Buurman (1977). The exine apertural membrane rupture in pollen of *Tabebuia pulcherrima* is a frequent phenomenon and always begins with the second volumetric expansion of the grain, apparently due to harmomegathic protrusion of the apertural membranes. Moreover, the exposed apertural membrane layer may be highly hydrophilic, due to the presence of pectic substances that enable the rapid water uptake and volumetric increase of the pollen grain, when in contact with the stigmatic fluid.

After being included by the cytoplasm of the vegetative cell, the generative cell approaches the nucleus of the former, at the same time the spherical shape of the generative cell changes gradually to become an elongated fusiform cell, with extremities often partially wrapped around the nucleus of the vegetative cell. These

observations show that, in mature pollen, an association exists between the generative cell and the nucleus of the vegetative cell, which is typical of the male germ unit in flowering plants. The male germ unit has been characterized and its physiological meaning interpreted by several authors (Russel & Cass 1981, Knox & Singh 1987, Rusche & Mogensen 1988, Pei-hua 1988, Mogensen 1992). In the present preparations, despite the clear association between the generative cell and the nucleus of the vegetative cell, it was observed in sections of mature polen grains that sometimes the generative cell can be separated from the vegetative cell nucleus by a short distance, suggesting that the generative cell can separate and return to associate with the nucleus of the vegetative cell, just as Pei-hua (1988) observed in relation to the sperm cells and the nucleus of the vegetative cell, during pollen tube development of *Amaryllis vittata* and *Clivia nobilis*. It has not been possible to confirm whether a real physical connection occurs between the generative cell and the vegetative cell nucleus of the pollen of *Tabebuia pulcherrima*.

The mature pollen grain in *Tabebuia pulcherrima* is bicellular, just as in *T. ochracea* (Bittencourt Jr. 1996) and in most of other Bignoniaceae (Ghatak 1956, Davis 1966, Gupta & Nanda 1978a, b, Mehra & Kulkarni 1985, Johri *et al.* 1992). However, Mehra & Kulkarni (1985) reported tricellular pollen grains in *Stereospermum chelonoides* and *Tabebuia rosea*.

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