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Ultrastructural studies on bicellular pollen grains of *Tillandsia sekeriana* Mez (Bromeliaceae), a neotropical epiphyte

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Abstract — The ultrastructure and ontogeny of bicellular pollen in *Tillandsia sekeriana* Mez (Bromeliaceae) before anther dehiscence has been here investigated. The development, after the first mitosis, is described to consist in three stages. The aim of the present report, which is part of a more extensive study on the reproductive mechanisms in the genus, is to compare the development of the male gametophyte of Tillands with information available from other angiosperms. Aspects on water, starch and soluble carbohydrate contents of the pollen of *T. sekeriana* are discussed in the light of the current ecological classification of pollens. The generative cell did not show any plastid in its cytoplasm, hence plastid inheritance in *Tillandsia* is confirmed as totally maternal (Lycopersicon type). Contrarily to other pollen grains, those of *T. sekeriana* maintain a considerable amount of starch in the vegetative cell until dehiscence. The inulin test was negative, indicating that *T. sekeriana* pollen lacks of Fructans. Pollen only with starch is subjected to faster dehydration and, probably, cannot be transported at long distance. The ecological adaptive plasticity of genus *Tillandsia*, is confirmed also by morphological characters that appear to be typical both of the partially dehydrated and partially hydrated pollen in *T. sekeriana*. This situation is, probably, a plesiomorphic character that permitted to genus *Tillandsia* to differentiate species well adapted to arid environments and others living in mesic forests.

Key words: pollen development, starch, *Tillandsia sekeriana*, ultrastructure.

INTRODUCTION

For many biological aspects the genus *Tillandsia* shows some of the most interesting characters in the Bromeliaceae, thanks to its pronounced epiphytism, based on peculiar absorbing trichomes. The mode of sexual reproduction can be considered an important factor in relation to the high number of *Tillandsia* species, some with very wide geographical distribution, other living in a restricted territory. This relationship justifies our interest in the reproductive apparatus of such neotropical epiphytes which has been matter of other previous studies (FRANCINI-CORTI 1981; BRIGHIGNA and PAPINI 1997; HESS 1991; SAJO *et al.* 2005). Recently these plants have reached a high economical importance, therefore their pollen has been studied for practical aims (PARTON *et al.* 2002). This research is a part of a more general study about pollen development in Tillands in relation to the colonized habitats.

In particular, the present work takes into consideration the ultrastructural changes before anther dehiscence of the male gametophyte in *Tillandsia sekeriana* Mez, a mesic species, in order to clarify its development and behaviour.

MATERIALS AND METHODS

Anthers of *Tillandsia sekeriana* were carefully removed from plants growing in a greenhouse of the Botanical Garden in Florence. In order to identify successive developmental stages of the bicellular pollen grain, the anthers were selected from buds at different developmental stages in the spike, and fixed as below.

LIGHT MICROSCOPY (L.M.) - The fructans were tested in polarized light. Inulin test (PACINI 2000) was done according to JOHANSEN (1940).

TRANSMISSION ELECTRON MICROSCOPY (T.E.M.) - Anther pieces, about 2 mm long, were fixed in 2.5% glutaraldehyde in phosphate buffer 0.1 M, pH 6.9 for 24 hr and then post-fixed in 2% OsO₄ in the same buffer for 2 hr.

After dehydration in an ethyl alcohol series, the samples were embedded in epoxy resin

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(SPURR 1969). Ultrathin sections obtained with a Reichert OMU 3 ultramicrotome were stained with uranyl acetate (GIBBONS and GRIMSTONE 1960) and lead citrate (REYNOLDS 1963), then observed using a Philips EM 300 TEM at 80 KV.

RESULTS

We divided the development of the male gametophyte in three stages on the basis of ultrastructural observations.

I – The small generative cell was still attached to the intine of the pollen grain and it showed an undulated convex wall (Fig. 1). This cell showed a large and regularly shaped nucleus with some peripheral chromatin aggregates (Fig. 2). In the cytoplasm, several small vacuoles, few mitochondria, and some dictyosomes occurred (Fig. 3). Rough endoplasmic reticulum (RER) elements were scarce, mostly arranged around the vacuoles. Plastids were lacking.

The large vegetative cell occupied the remaining volume of the grain. It had a roundish nucleus with a huge nucleolus and finely dispersed chromatin (Fig. 4a). The vegetative cytoplasm was richer in organelles than the generative cell, showing many leucoplasts with starch grains and many vacuoles containing dispersed material (Fig. 4). The most frequently observed organelles in the peripheral cytoplasm were mitochondria with evident cristae and a little electron dense matrix, clustered dictyosomes with few cisternae and tiny vesicles (Fig. 5) and long ER cisternae (Fig. 6).

II – The generative cell appeared free in the mature grain (Fig. 7). The process of detachment from the intine started with the progressive narrowing of the generative cell wall. This mechanism allowed the release of the cell inside the vegetative cytoplasm and was demonstrated by the presence of a residue, which was deeply attached to the intine (Fig. 8). The generative cell assumed a roundish shape and was surrounded by lipid droplets (Fig. 7). Its wall was irregularly thin.

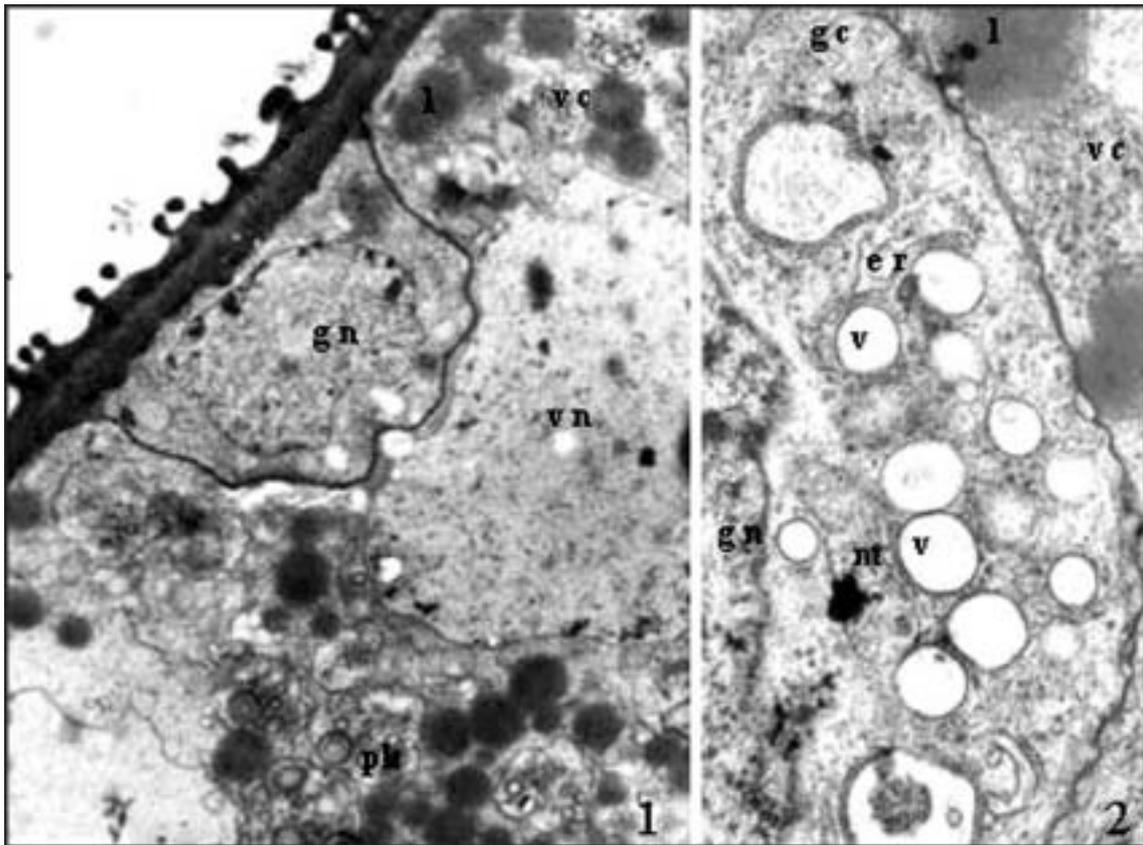


Fig. 1-2 — *First stage*. (v, vacuoles; gc, generative cell; vc, vegetative cell; 1, lipid droplet; W, wall; gn, generative nucleus; m, mitochondria; rer, rough endoplasmic reticulum; pl, plastid). Fig. 1. The generative cell is still attached to the inner layer (intine) of the grain. x 6,000. Fig. 2. Generative cell cytoplasm detail: little vacuoles bordered by rough endoplasmic reticulum elements and mitochondria with few cristae are present. x 6,500.

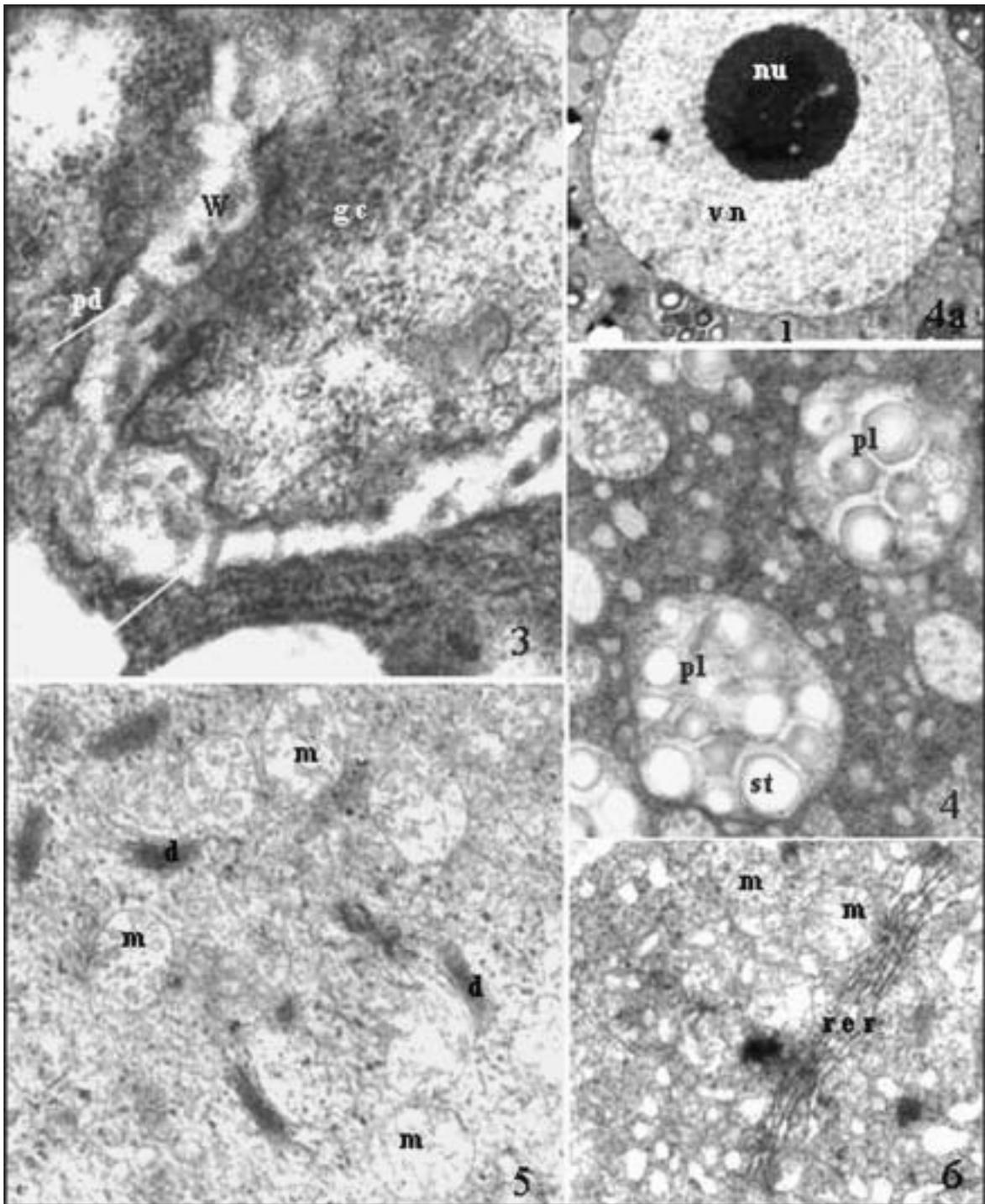


Fig. 3-6 — *First stage*. (v, vacuoles; gc, generative cell; vc, vegetative cell; l, lipid droplet; W, wall; vn, vegetative nucleus; m, mitochondria; d, dictyosomes; pl, plastid; nu, nucleolus; rer, rough endoplasmic reticulum). Fig. 3. Convex wall portion of the generative cell: the wall is very irregular in thickness, alternating thin tracts to thick ones. In the generative cytoplasm tiny vacuoles are shown while the vegetative cytoplasm appears denser and rich in organelles. x 17.200. Fig. 4a. Detail of the vegetative nucleus that is voluminous and roundish with a huge and spongy nucleolus. x 3.900. Fig. 4. Vegetative cytoplasm rich in storage plastids filled mostly by starch grains. x 11.000. Fig. 5. Detail of the peripheral vegetative cytoplasm where mitochondria with evident cristae and low dense matrix and dictyosomes budding inconspicuous vesicles occur. x 18.100. Fig. 6. The vegetative cytoplasm appears often crossed by long and parallel RER strands. x 15.300.

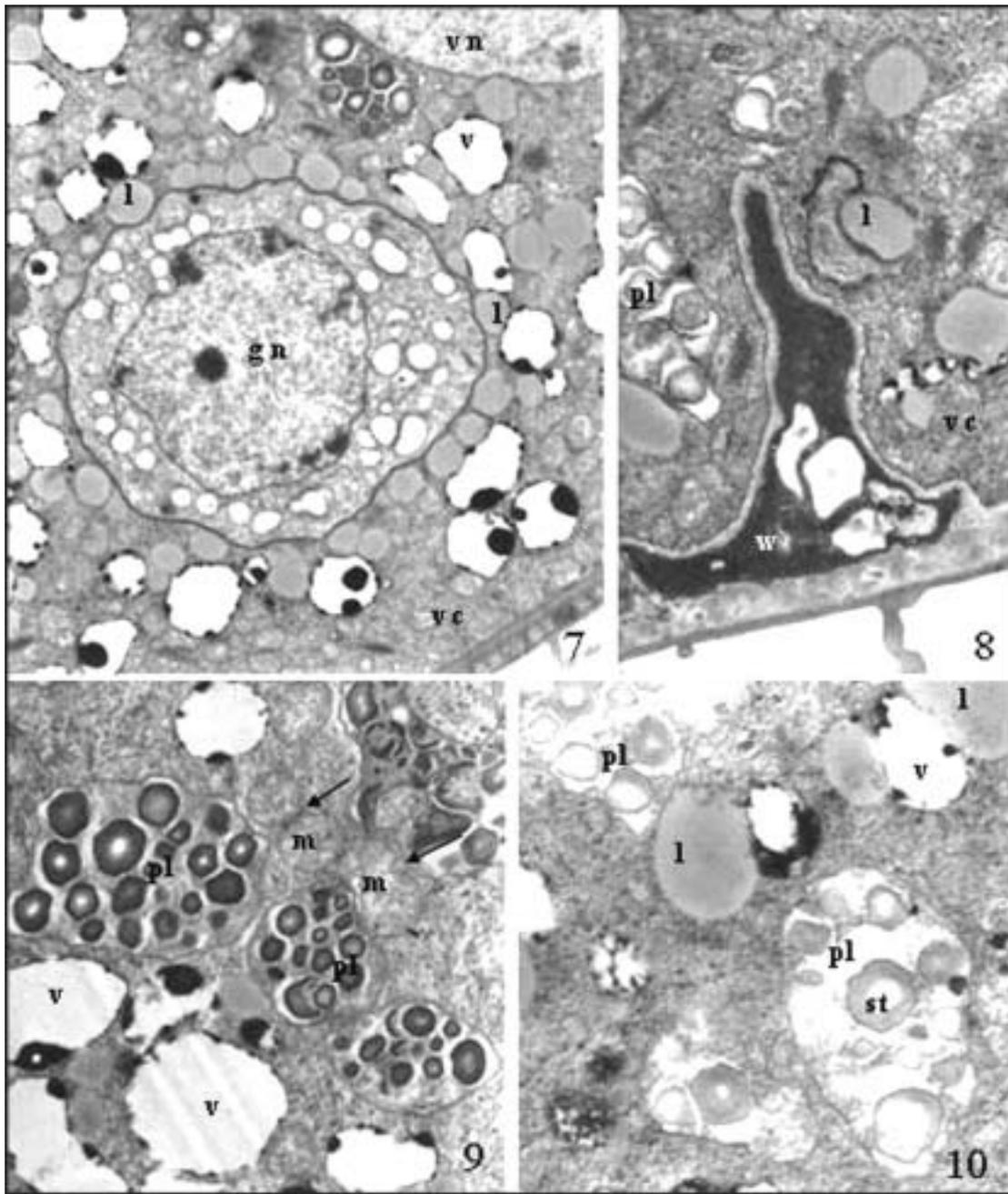


Fig. 7-8 — *Second stage*. (v, vacuoles; gc, generative cell; vc, vegetative cell; l, lipid droplet; W, wall; gn, generative nucleus; vn, vegetative nucleus; m, mitochondria; d, dictyosomes; pl, plastid; nu, nucleolus; i, intine). Fig. 7. The generative cell is now detached from the intine and appears roundish and free in the vegetative cytoplasm. The gc is typically surrounded by lipid droplets and has a regularly outlined nucleus with peripheral chromatin aggregates. The vegetative cytoplasm appears denser and rich in plastids, mitochondria, lipid droplets and vacuoles. $\times 5,900$. Fig. 8. After the release of the generative cell, exactly in the point where it was attached to the intine, an electron-dense residue persists. In the vegetative cytoplasm mitochondria, plastids, dictyosomes and lipid droplets are shown. $\times 7,200$.

Neither the vegetative nucleus changed its shape nor the disposition of chromatin varied (Fig. 4a); on the contrary considerable variations appeared in the vegetative cytoplasm where the fragmentation of plastids into little units oc-

curred. A progressive reduction in starch content of plastids started. At the same time the vacuoles were in contact with lipid droplets and leucoplasts; furthermore vacuoles showed irregular aggregates of strongly osmiophilic material adhering

to the tonoplast and occasionally myelin-like bodies (Fig. 10). Mitochondria, some of them in division phase, were abundantly distributed in the peripheral cytoplasm (Fig. 9).

III – The generative cell assumed a club shape. Its main part contained the nucleus, now irregularly shaped, and many organelles, including vacuoles surrounded by RER, mitochondria with few cristae and some dictyosomes. Transverse and longitudinal cell sections (Fig. 11 and Fig. 13) showed an irregularly scalloped outline of the thin envelope; in some tracts, it was so reduced in thickness that the two plasma membranes seemed to be in mutual contact. A relevant feature of this stage was the high number of microtubules radially disposed around the nucleus (Fig. 12) and in parallel orientation with respect to the main cell axis in the remaining tail (Fig. 13).

In the vegetative cytoplasm the nucleus appeared deeply lobed and surrounded by con-

spicuous and concentric RER bodies (Fig. 14 and Fig. 14a). The plastids, devoid of starch, became elongated and amoeboid, with an osmiophilic matrix (Fig. 15); they were associated to long RER profiles.

At polarized light microscope the pollen grains resulted lacking of inulin spherocrystals (Fig. 16).

DISCUSSION

During pollen maturation the generative and the vegetative cells undergo ultrastructural modifications that underline their different destiny; the vegetative cell provides nourishment to the developing generative cell and accumulate material necessary for pollen germination. On the basis of morphology and different positions assumed by

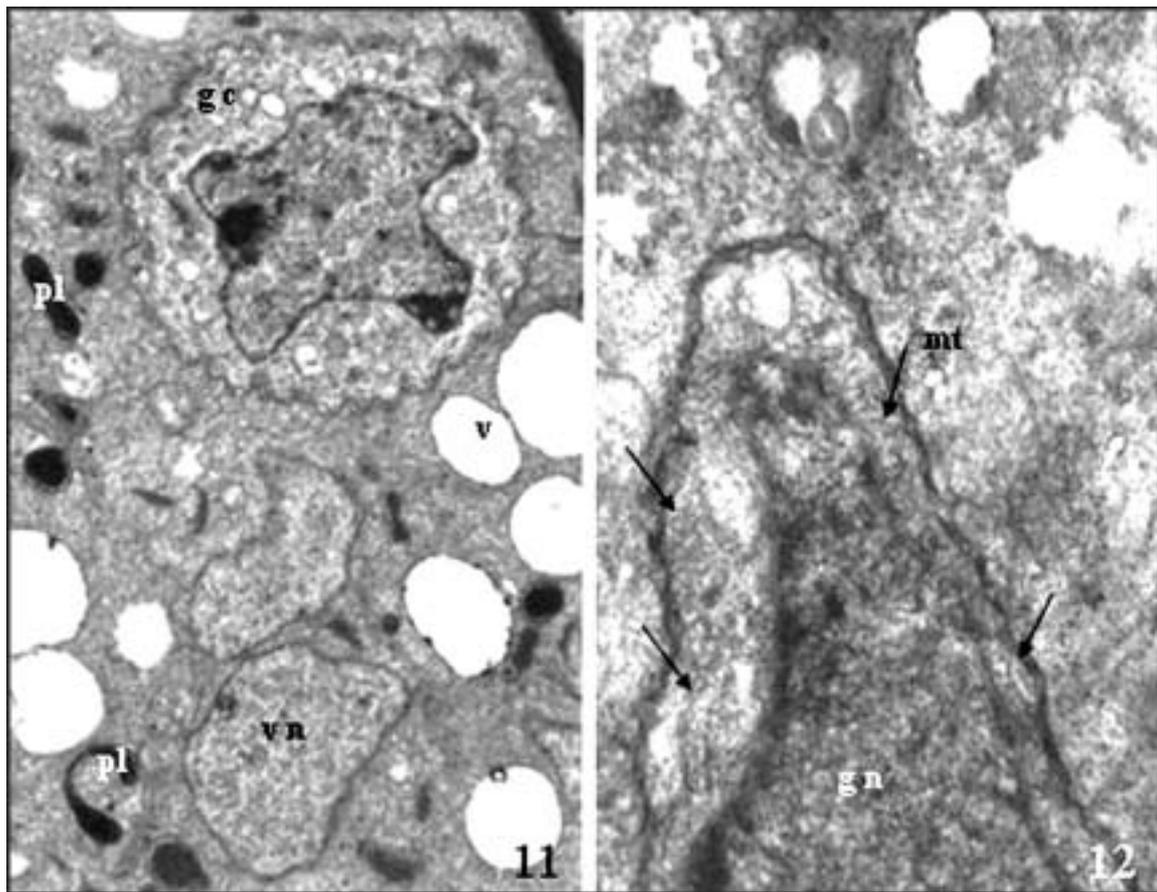


Fig. 11-12 — *Third stage*. (v, vacuoles; gc, generative cell; vc, vegetative cell; l, lipid droplet; gn, generative nucleus; mt microtubules; d, dictyosomes; pl, plastid). Fig. 11. The vegetative cytoplasm that surrounds the generative cell is rich in plastids now devoid of their glycidic reserves. They appear amoeboid and with a strongly osmiophilic matrix. Vacuoles are now lacking electrondense content. x 9.900. Fig. 12. Detail of a transversally cut generative cell: the microtubules (arrows) are radially arranged; the nucleus is deeply lobed with chromatin aggregates; the wall is thin. x 25.500.

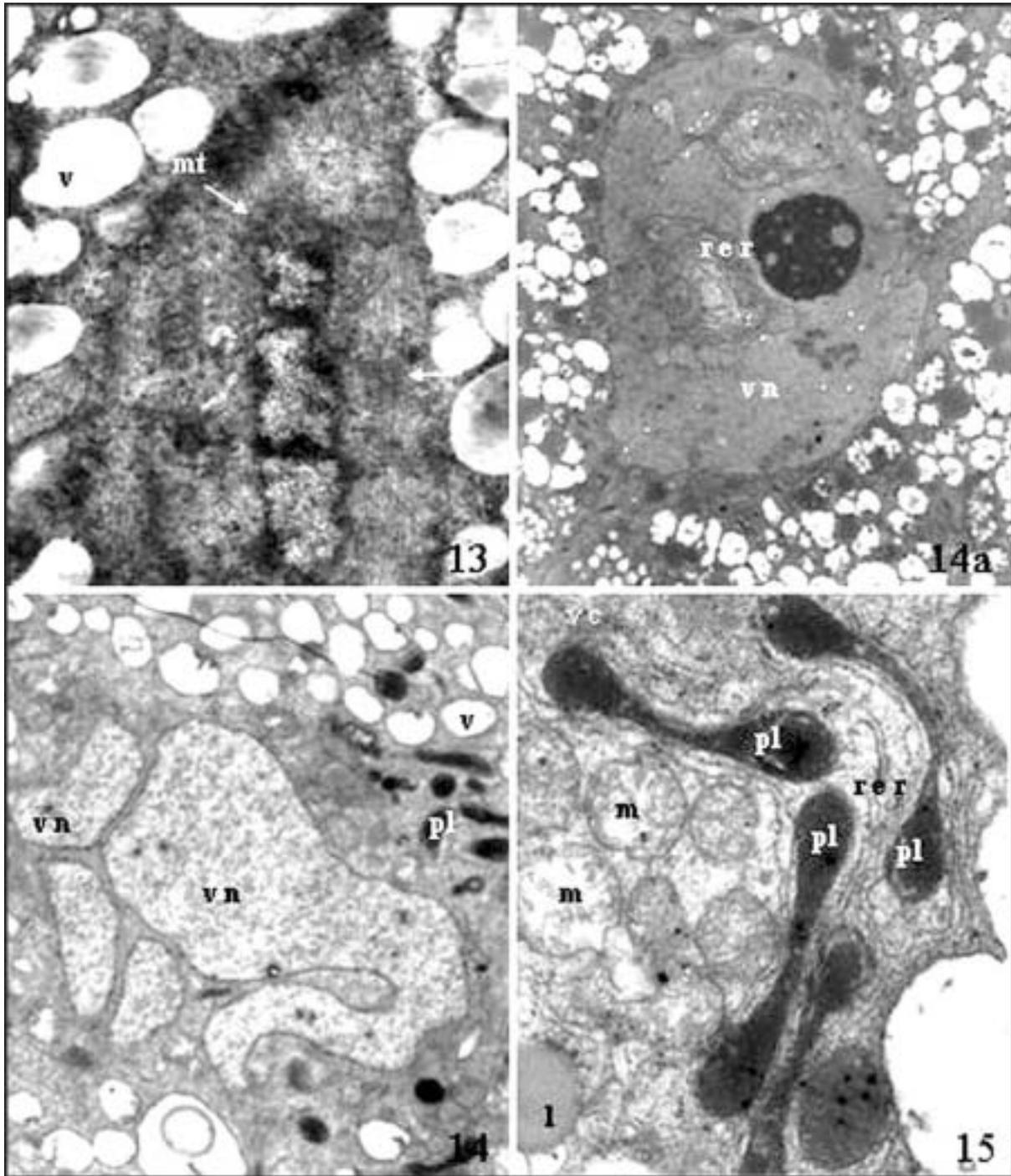


Fig. 13-15 — *Third stage*. (v, vacuoles; gc, generative cell; vc, vegetative cell; l, lipid droplet; vn, vegetative nucleus; mt microtubules; d, dyctiosomes; pl, plastid; rer, rough endoplasmic reticulum). Fig. 13. Portion of longitudinally cut generative cell: microtubules run parallelly to the longitudinal axis of the cell. Inside the generative cell essentially small vacuoles and mitochondria with few cristae occur. $\times 11,700$. Fig. 14a. Concentric tangles of RER find place inside the deep pockets of the polymorphous vegetative nucleus. $\times 5,000$. Fig. 14. Extraordinarily lobed vegetative nucleus with finely dispersed chromatin. In the vegetative cytoplasm lots of ameboid plastids, mitochondria and small vacuoles are present. $\times 8,200$. Fig. 15. Vegetative cytoplasm with thin, ameboid plastids with strongly electron-dense content profiled by RER. Vacuoles lacking osmiophilic material inside are present. $\times 29,700$.

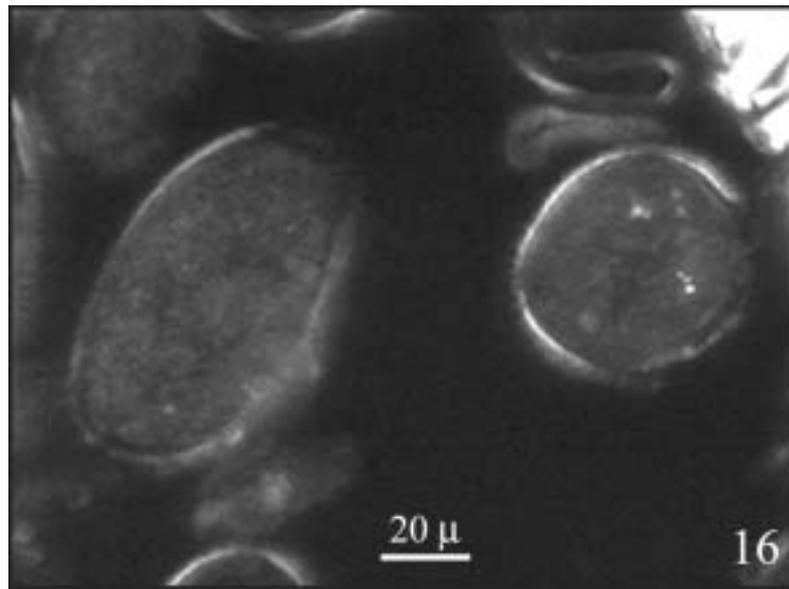


Fig. 16 — At the polarized light microscope the pollen grains resulted lacking in inulin spherocrystals.

the generative cell in the vegetative cytoplasm we have identified three stages of male gametophyte development before anther dehiscence. These stages show evidence of high activity of the vegetative cell. Also the modifications in shape of the generative cell are distinctive features. At the beginning, the two cytoplasms are different as a consequence of the asymmetric mitotic division in the single celled microspore (ANGOLD 1968; HESLOP-HARRISON 1968; Mc CORMICK 1993). The most evident difference is that the vegetative cytoplasm appears rich in amyloplasts while the generative cell is substantially poor in organelles and lacks plastids. The metabolic activity is hence primarily carried out by the vegetative cell till the release of the generative cell into the cytoplasm of the vegetative cell. The absence of plastids in the generative cell cytoplasm places *T. seleriana* in the *Lycopersicon* type (SCHROEDER 1984; 1985), the most common in angiosperms. Plastids have been shown in the generative cell of *Lilium* (BOPP-HASSENKAMP 1960), *Oenothera hookeri* (DIERS 1963) and in other plants of several families (see LARSON 1963; MOGENSEN 1996). In *Tillandsia* the inheritance of plastids appears to be completely maternal. The intense biosynthetic activity that takes place in the vegetative cell of the young gametophyte of *T. seleriana* is indicated by numerous mitochondria, ribosomes and RER arrays. The observed morphological features are similar to those seen in other Bromeliads (FRANCINI-CORTI 1981; BRIGHIGNA *et al.* 1981) and other angiosperms as *Nicotiana glauca* (CRESTI *et al.* 1985). The vegeta-

tive cell adjusts its metabolic activities in order to lose water, which immediately results in the dehydration of the pollen grain. This phenomenon is confirmed by the decrease of the vacuole dimensions. Clustered dictyosomes and their budding vesicles are involved in the completion of the pollen wall.

The abundance of amyloplasts in the vegetative cytoplasm suggests its important trophic function in regard to the generative cell. After callose elimination and the following release of microspores from the tetrad, the vegetative cytoplasm starts to store carbohydrates. The meaning of this storage can be explained only by analyzing the entire maturation phase of pollen grain development. In the first stage the generative cell forms after the mitosis and remains attached to the inner layer of the grain wall preserving plasmodesmata with the sister vegetative cell.

In the second stage the generative cell gets detached from the intine assuming mobility in the vegetative cell. The acquired freedom of the generative cell causes the loss of the symplastic connections between the two cells. According to HESLOP-HARRISON (1968) the elimination of the cytoplasmic bridges occurs during the migration of the generative cell in the pollen tube. A reduction of the exchanges between the two cytoplasms is the likely effect. Those exchanges, however, do not seem to be entirely interrupted, since the wall electron density appears increased in its thinnest tracts. This observation agrees with that by BRIGHIGNA *et al.* (1981) who observed, during the

third stage of development of *T. caput-medusae* pollen, the transit of electron dense material through narrow channels of the generative cell wall. These authors have interpreted this fact as an indication that among the two cells some exchanges are necessary and that the loss of plasmodesmata do not cause the total isolation of the two cytoplasm. The vegetative cytoplasm shows the amyloplast fragmentation and a progressive reduction of their polysaccharide reserves. The emptying of these organelles indicates that starch is hydrolyzed. This process leads maltose to be exported from plastids to the cytoplasm where it will be converted to glucose and eventually to other carbohydrates (SMITH *et al.*, 2005). Also the persistence of RER associated with the vacuoles which envelope the lipid droplets suggests the involvement of such substances in the formation of the material stored in the vacuoles. The appearance of this material is connected to the substances necessary for the rapid growth of the pollen tube at germination time. COCUCCI (1969) indicated that the osmiophilic material present in the vegetative cell vacuoles of *Epidendrum scutella* pollen is used for such function. From the metabolic point of view, the abundance of mitochondria in the zones where these intense activities occur is meaningful and supports our explanation. Another modification in the generative cell is the general thinning of the wall that supports the plasticity needed to assume an elongated shape. The fusiform club shape will facilitate the transit through the narrow pollen tube.

The third stage is characterized by the lengthening of the generative cell. This process is realized and regulated by the formation of numerous microtubules. The functional role of microtubules as cytoskeletal structure has been shown in *Gossypium hirsutum* (JENSEN *et al.* 1968; 1970) and *Haemanthus katherinae* (SANGER and JACKSON 1971). The presence of fibrous bodies has been related to the production of microtubules in the generative cell of *T. caput-medusae* (BRIGHIGNA *et al.* 1980b). Microtubules associated with fibrous bodies have been localized in the vegetative cytoplasm of *Clivia* and *Lilium* (FRANKE *et al.* (1972) and *Petunia hybrida* (CRESTI *et al.* 1976). In these two cases the microtubules did not have only a cytoskeletal role, but they were also strictly related to the movements inside the cells as indicated also by other authors (PARTHASARATHY and MÜHLETHALER 1972).

The deeply lobed shape of the vegetative nucleus indicates an intense metabolic activity of the mature vegetative cell. The abundance of periph-

eral dictyosomes may be related with the completion of the inner layer of the grain wall. The activity operated by the elements of RER towards the plastids is in agreement with the intense cellular metabolic activity, as well as the enrichment in ribosomes of the cytoplasmic matrix. The association of RER and plastids is a very prominent feature in the pollen grain of *T. seleriana*. According to CRESTI *et al.* (1977) this association is an evidence of mutual exchange of substances between RER and plastids. CRESTI and PACINI (1976) suggested two possible meanings for this association: a) the proximity between RER and plastids would help the direct exchange of soluble carbohydrates from and to the plastids; b) a mechanism for the synthesis of molecules used both inside and out of the plastids. However, in *T. seleriana* the ultrastructural details suggest the disintegration of these organelles. The disappearance of plastids is a preparatory phase for the pollen grain germination events, a process that requires the development of a large vacuole in the vegetative cell at the pole opposite to the point of emergence of the pollen tube, and the tube plasmamembrane construction (BRIGHIGNA and PAPINI 1997).

In conclusion, the pollen grain of *T. seleriana* shows maturation stages before anther dehiscence which are not substantially different from those generally known in flowering plants (resumed by MAHESHWARI 1950; HESLOP-HARRISON 1968; ESAU 1977). Nevertheless, *T. seleriana* shows a particular feature: it presents an extended two-cell phase that delays the formation of the sperm cells. Such prolonged two-cell phase can also be observed in a lot of tropical orchids (SWAMY 1949; COCUCCI 1973). However the two-celled prolonged condition does not seem related to the chemical form under which the reserve substances are present, since *T. seleriana* has a considerable amount of starch reserve in the vegetative cell while such storage is absent in that of orchids (LARSON 1965; CHACHARD 1969; COCUCCI and JENSEN 1969). During the maturation of the male gametophyte the reserve substances and their reversible transformation between osmotically active or inactive molecules, are very important and leads to meaningful consequences for pollen ecology (PACINI 1994). On the basis of their carbohydrates reserves, pollen grains have been classified in three groups (FRANCHI *et al.* 1996; SPERANZA *et al.* 1997): I) only with starch, II) with starch and cytoplasmic sugars, III) only with cytoplasmic sugars. Pollen belonging to the first category is quickly dehydrated and loses the ability to be transported at long distance (BARNABAS 1985;

NEPI and PACINI 1993). Pollen with cytoplasmic sugars is long living and resists better to dehydration. In other words, the presence of cytoplasmic carbohydrates and the abundance of saccharose are correlated with the longevity of the pollen grain.

In particular sucrose protects the integrity of membranes during dehydration guaranteeing the functionality of their proteins (HOEKSTRA *et al.* 1992; SPERANZA *et al.* 1997). About the mechanism with which the sucrose carries out this function, FRANCHI *et al.* (1996) showed that this disaccharide is intercalated among the polar heads of the phospholipids replacing the water molecules lost during dehydration. HOEKSTRA and VAN ROEKEL (1988) observed in the pollen of *Papaver dubium* a notable increase in saccharose content during the three days before anthesis; this increase was related to the acquisition of the ability to tolerate desiccation after germination.

Pollen classification by FRANCHI *et al.* (1996) and SPERANZA *et al.* (1997) is the base of the most recent observations of PACINI (2000). This author recognized two categories of pollens: a) with fructans and saccharose; b) without or scarce fructans and saccharose. Fructans and saccharose derive from the total or partial hydrolysis of the starch contained in the pollen grain before anther dehiscence. From this point of view a direct relationship has been proposed between the degree of hydrolysis of the pollen starch and the quantity of cytoplasmic sucrose (PACINI 2000). Pollens with saccharose and fructans have mechanisms able to regulate their water content and to withstand thermal shocks. These pollens have a furrow to help water exchanges and can survive to fluctuations of temperature and humidity. On the contrary, pollens with scarce or absent fructans and sucrose do not have mechanisms able to regulate water exchange, nor to withstand thermal shock. They lack furrow and survive better in places where humidity is high and temperature is low.

The negativity of the inulin test demonstrates that *T. seleriana* pollen lacks fructans. Despite this, the initial great amount of starch in the pollen of the examined species and its following disappearance should cause the increase of cytoplasmic sugar concentration. On the other hand we cannot exclude the presence of sugar linked to agluconic groups (glycosides) inside the vacuoles as substances which would not be detectable with inulin test.

According to current pollen classification, that considers as main character the water content (NEPI *et al.* 2001), the pollen of *T. seleriana* shows

characters of both categories (pollens partially dehydrated (PD) and pollens partially hydrated (PH). These data may have an ecological meaning, indicating a high adaptive plasticity that could be one of the reasons of the many different habitats where Tillands live. This pollen character is, probably, a plesiomorphic character that permitted to the genus *Tillandsia* to differentiate species well adapted to arid environments and others living in mesic forests.

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