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Characterization of the seed reserves in *Tillandsia* (Bromeliaceae) and ultrastructural aspects of their use at germination

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Abstract — The nature and the use of the reserves accumulated in the seed of *Tillandsia*, a markedly epiphyte genus, are especially important in consideration of the subsequent seedling development. As a matter of fact, the embryo radicle degenerates precociously without emerging at the germination and the sole function of the root system is that of anchoring to the substratum.

The present cytochemical and ultrastructural study carried out on mature seeds has highlighted the presence of proteins and lipids, in both the endosperm and in the cotyledon, which are both well-developed; in addition, the endosperm contains starch and calcium oxalate. At germination the reserves, broken-down by enzymes, are absorbed by the cotyledon in whose epidermis ultrastructural modifications, correlated with this austorial function, appear. A part of the nutrients absorbed is initially stored in the large vacuoles, of aleuronic origin, of the cotyledon parenchyma. The result is a water uptake. It is in this manner that the water and necessary nutrient needs of the seedling are satisfied during early development.

Key words: germination, seed, *Tillandsia.*

INTRODUCTION

The genus *Tillandsia* is characterized by an intense epiphytism even in extreme environments (MEZ 1904); this life condition makes these plants take on morpho-physiological adaptations suitable to a rigorous economy of wa ter and nutrients. The absorption of these is performed by highly specialized foliar tri-chomes (BENZING 1980; FRANCINI 1981), while the adventitious root system has as its sole func tion the task of anchoring to the substratum (BRIGHIGNA *et al.* 1990). These features are especially pronounced in the species which are defined as being "atmospheric" (MEZ 1904). From this point of view, the nature of the reserve substances stored in the seed and their use take on a particular importance for the success of the seedling development at least until the special absorbing system becomes differentiated. The ultrastructural data available in the bibliography refer mainly to the seeds of dicotyledons and of some monocotyledons amongst which the Graminaceae.

The present study examines the seed df some atmospheric species of *Tillandsia* in order to carry out a cytological analysis of the location of the reserves, their chemical nature and the embryo parts mainly involved in the use of the very reserves at germination.

MATERIALS AND METHODS

For the present study, seeds of *Tillandsia juncea, T. fasdculata, T. complanata* taken from specimens collected in Mexico, Costarica and Peru and cultivated in the greenhouses of the Botanical Garden in Florence, were used.

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To identify the location as well as the chemical nature of the reserves, some seeds from dehiscent capsules were fixed and included for observation using the Light Microscope (L.M.) and the Transmission Electron Microscope (T.E.M.). In order to study the use of the reserves, other seeds were set in a moist room to germinate, in alternating day/night conditions and at room temperature. The samples for L.M. and T.E.M. observations were carried out after 7, 15, and 30 days. All the seeds were deprived of the dispersion system to reduce the growth of saprophytes during germination.

Light Microscopy (L. M.)

Mature and germinating seeds (the latter sampled as mentioned above) were treated with the following fixatives: FAA QENSEN 1962) for 10 days, Bouin's fluid (BECCARI and MAZZI 1966) for 14 days, formalin-calcium QENSEN 1962) for 24 hours.

Following dehydration in ethyl alcohol, the embedding was carried out in paraffin. The difficulty related to sectioning did not permit us to get sections less than 8-10|im thick. A Reichert-Jung microtome was used. Cytochemical reactions were carried out using different types of staining in an aqueous solution and in an alcohol solution. The presence of the following compounds was tested: Neutral polysaccharides:

— White light: PAS reaction QENSEN 1962)
— Fluorescence: Acriflavine (O'BRIEN and Mc-CULLY 1981)
— Polarized light: Crossed Nicols (EVERSON PEARSE 1972).

Lipids:

— White light: Sudan Black; Sudan III and Sudan IV (BECCARI and MAZZI 1966)
— Fluorescence: Fluoral Yellow 88 (BRUNDRETT *et al.* 1991) on Cryo-Cut microtome freshly sectioned material (sections 40µm thick). Sections were stained for 30 minutes.

Proteins:

— White light: Mercury-bromophenol blue (EvERSON PEARSE 1968).

Calcium oxalate:

— White light: Chalk reaction QENSEN 1962)
— Polarized light: Double refraction (BUTTROSE and LOTT 1978); X ray lamina (chalk), - X lamina (mica).

Protease:

— Fluorescence: Bodipy FL (E-6638) (TWINING 1984).

A Leitz D.M.- R.B. L.M. was used for white light, polarized light and fluorescence observations. With reference to fluorescence observation, the spectrum shift, observed at times, may be due to a partially homogeneous chemical nature of the substratum with which the fluorochrome bonds (EVERSON PEARSE 1972).

Transmission Electron Microscopy (T.E.M.)

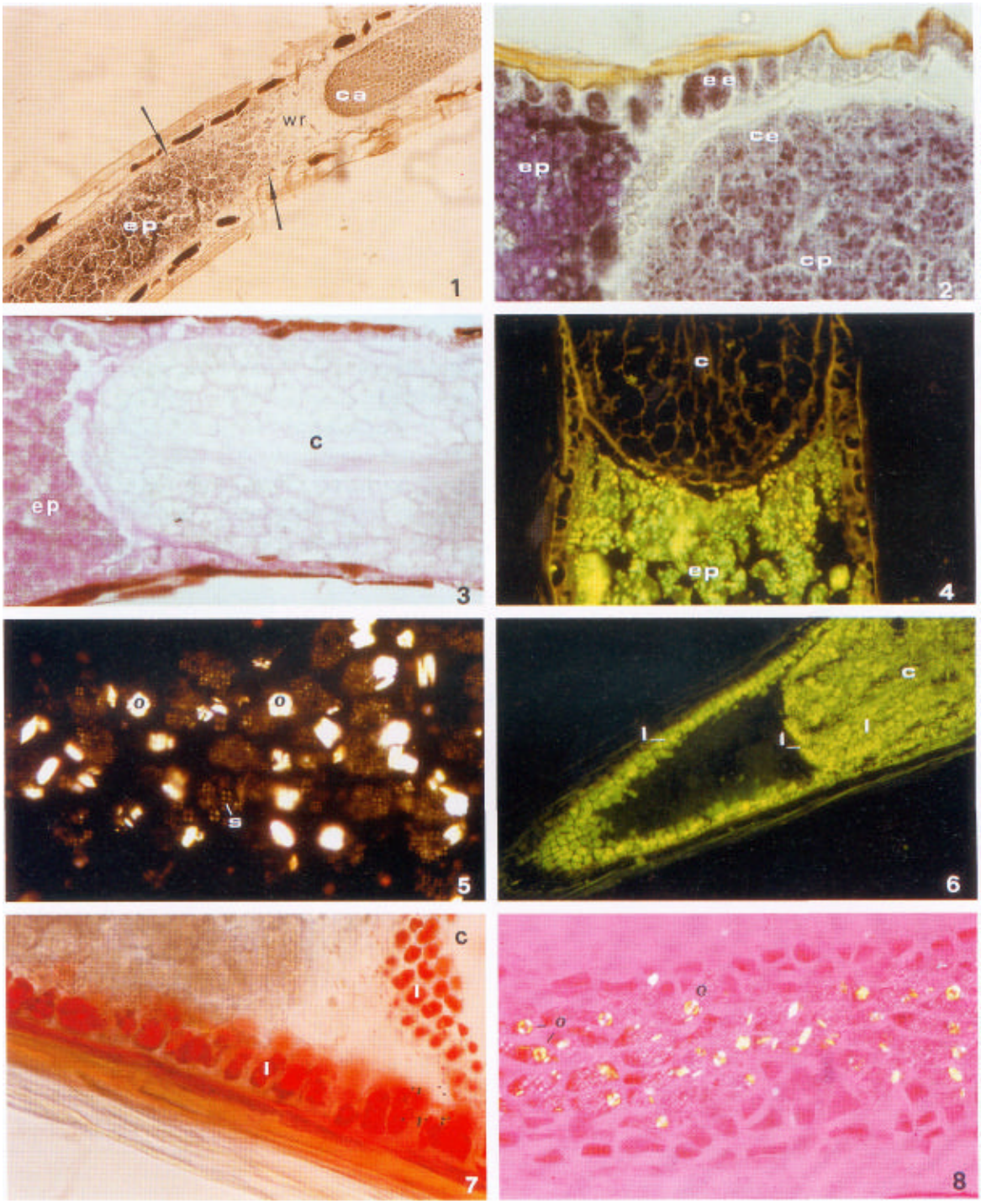
Mature seeds and ones at the above mentioned different stages of germination were stripped also of their outer integument as well as of the more micropylar and chalazal portions to facilitate the fixative and inclusion media penetration. These seeds were fixed in a glutaraldehyde and paraformaldehyde mixture in phosphate buffer 0.05M, pH 7.2 at 3°-4° C for 18 hours. After rinsing in the same buffer, the samples were post-fixed in OsO4 2% for 2 hours. The inclusion was carried out in Spurr's resin (SPURR 1969).

Ultrathin sections obtained with a Reichert OM U3 ultramicrotome were contrasted with uranyl acetate (WATSON 1958) for 2 hours and with lead cytrate (REYNOLDS 1963) for 2 minutes.

The micrographs were carried out using a Philips EM-300 T.E.M. The presence of acid phosphatase was tested in samples taken at day 7 of germination, using the Gomori method according to Poux (1970).

Abbreviations: a: aleurone grain; c: cotyledon; **ca:** cotyledon apex; ce: cotyledon epidermis; **ch:** chloroplast; **cp:** cotyledon parenchyma; e: endomembranes; **ee:** endosperm epidermis; **em:** electrontransparent material; **en:** endosperm; **ep:** endosperm parenchyma; g: glyoxysomes; 1: lipids or spherosomes; m: mitochondrion; o: calcium oxalate; p: proteins; s: starch; v: vacuole; **ve:** vesicle; w: wall; **wr:** wall remnants.

Figs. 1-18: Light Microscopy. Fig. 1 — Portion of mature seed in longitudinal section. The endosperm epidermis (arrows) is single-layered; the endosperm parenchyma has many reserve substances; beside the cotyledonar apex, it is made up only of diaphanous wall remnants. Stained with Ferrous haematoxylin (70 x).Fig. 2 — Portion of mature seed in longitudinal section. The epidermis and the parenchyma both of the endosperm and of the cotyledon are rich in protein reserves. Stained with Mercury-bromophenol blue (300x).Fig. 3 — Portion of mature seed in longitudinal section. All the cell walls are PAS positive, just as the abundant starch granules of the endosperm parenchyma (140x).Fig. 4 — Portion of mature seed in longitudinal section. Staining with Acriflavine confirms the PAS reaction results (140x).Fig. 5 — Mature seed. Portion of endosperm parenchyma: the observation in polarized light shows the dark cross of the starch granules and the double refraction of the calcium oxalate (420x).Fig. 6 — Portion of mature seed in longitudinal section. The fluorescence due to Fluoral yellow 88 staining indicates the presence of lipids in the endosperm epidermis and in the cotyledon (7Ox).Fig. 7 — Portion of mature seed. Staining with Sudan III and IV confirms the presence of lipids in the endosperm epidermis and in the cotyledon (300x).Fig. 8 — Mature seed. Portion of endosperm parenchyma: the observation in polarized light with A, wave lamina/sheet (chalk) and - A, wave lamina/sheet (mica) shows the Maltese Cross typical of the calcium oxalate crystals (300x).



RESULTS

Light microscopy

Mature seeds

The observations of longitudinal sections of mature seeds of the species of *Tillandsia* studied show that the endosperm is made up of a mass of parenchyma tissue lined on the outside by a single layer of living cells which have a compact arrangement form in a sort of epidermis. In the seed more micropylar region, at the level of the hypocotyl/embryo radicle axis and of the cotyledon lateral surfaces, the epidermal layer is the sole portion of the endosperm which can be clearly identified. Between the endosperm epi-

dermis and the embryo, the endosperm parenchyma is reduced to a series of tightly pressed together cell walls. In the seedintermedial portion the cotyledon apical part is immersed in the endospermal parenchyma which also fills the seed more chalazal portion. The endospermal parenchyma is composed of cells rich in reserve substances; exactly in front of the cotyledon apex, the endospermal cells are reduced to only cell walls (fig. 1). The embryo is lined by a single layer of epidermis. The cotyledon is highly developed (fig. 1) and beneath the epidermis presents a parenchyma full of stored nutrients (fig. 2).

The cytochemical reactions carried out indicate the presence of proteins in all the tissues mentioned: endospermal epidermis and parenchyma, cotyledon epidermis and parenchyma (fig. 2). The PAS reaction is positive around the cotyledon, at the level of the tightly pressed walls, and especially where abundant starch granules are present in the endospermal parenchyma; both results are confirmed by the fluorescence of the staining withAcriflavine and by the observation with polarized light between crossed Nicols (figs. 3, 4, 5). The reactions carried out to highlight the lipids show their abundant presence in the endosperm epidermis. Lipids are also present in the cotyledon epidermis and parenchyma (figs. 6, 7).

In the endosperm parenchyma, the abundant presence of crystalline structures is observed; the specific chemical reaction and observation with the polarized light clarify their calcium oxalate nature (figs. 5, 8).

Germinating seeds

7 days — The seeds appear swollen when compared to the dry seeds, due to the fact that they have taken-up water during imbibition (figs. 9, 9a).

The longitudinal sections, treated with specific types of staining and observed with the L.M., show the same localization of abundant protein, polysaccharide, lipid and calcium oxalate reserves, as observed in the mature seed. A considerable presence of protease is found in the cotyledon epidermis, in the adjacent endospermal parenchyma and epidermis (fig. 10).

15 days — The seeds observed in toto show rupture of the inner integument at the hypocotyl level, which is swollen (fig. 11).

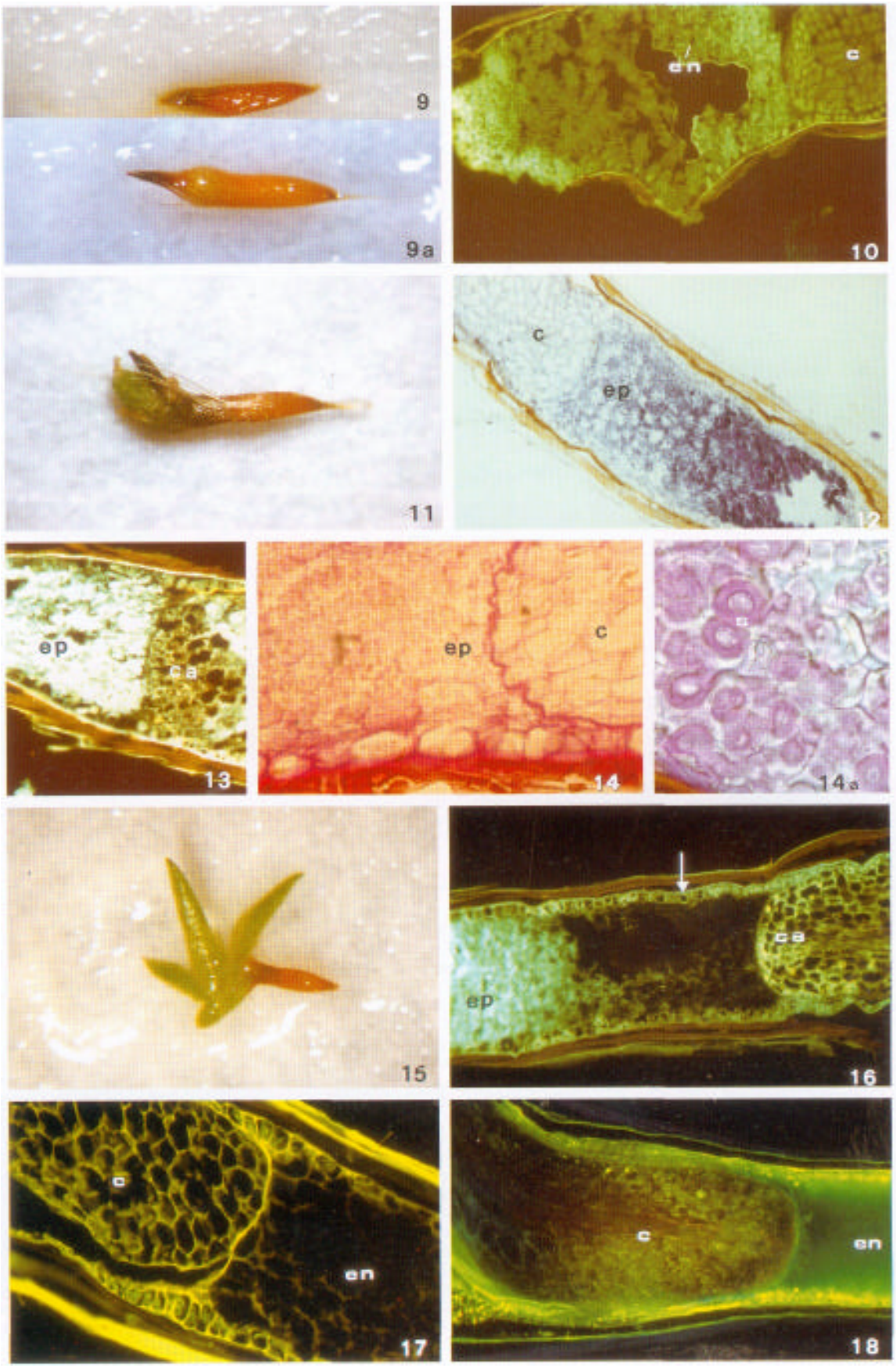
In the material observed at L.M., the specific stainings carried out indicate a reduction in the proteins mainly in the cotyledon and, to a lesser degree, in the endosperm parenchyma in front of the cotyledon apex (fig. 12); in the same endospermal region, the fluorescence due to the reaction caused by the protease remains evident (fig. 13).

In the endosperm parenchyma, the PAS positive starch granules are fewer in the portion in front of the cotyledon; in the *chalazal* parenchyma, the majority of the above granules show, in the sections, a ring morphology (figs. 14,14a) given the degradation of the central area. The response to the specific reaction appears unchanged for both the endosperm and cotyledon lipids, as does that of the calcium oxalate in the endosperm parenchyma.

30 days — The germinating seeds show the emergence of the first leaflets (fig. 15).

The longitudinal sections observed at the L.M. after being treated with the specific staining, highlight the further decrease in the protein reserves mainly in the endospern in front of the

Fig. 9 — Full view of a dry seed (lOx).Fig. 9a — Full view of a swollen seed (7 days) (lOx).Fig. 10 — Portion of germinating seed in longitudinal section (7 days). The fluorescence highlighted by the reaction with Bodipy indicates the presence of protease in the cotyledon and in the endosperm (7Ox). Fig. 11 —Full view of a germinating seed (15 days). Note the swollen hypocotyl and the rupture of the internal tegument (10 x).Fig. 12 — Portion of germinating seed in longitudinal section (15 days). The staining with Mercury-bromophenol blue indicates a decrease in proteins mainly in the cotyledon and, to a lesser extent, in the endosperm parenchyma in front of the cotyledon (70x).Fig. 13 —Portion of germinating seed in longitudinal section (15 days). The fluorescence due to the reaction for the proteases remains evident in the endosperm parenchyma which is in front of the cotyledon apex (90x).Fig. 14 — Portion of germinating seed in longitudinal section (15 days). The PAS reaction shows a reduction of the starch in the endosperm parenchyma in front of the cotyledon (420x).Fig. 14a — Germinating seed (15 days). Detail of the endosperm parenchyma showing the starch granules with ring-like morphology. PAS reaction (420x). Fig. 15 — Full view of the emerging seedling (30 days) (8x).Fig, 16 — Portion of germinating seed in longitudinal section (30 days). The fluorescence due to the reaction for the protease remains mainly in the endosperm epidermis (arrow), and in the chalazal endosperm parenchyma and, to a lesser degree, in the cotyledon apex (70x). Fig. 17 — Portion of germinating seed in longitudinal section (30 days). The fluorescence due to Acriflavine indicates the almost total disappearance of the endosperm polysaccharide reserves (140x).Fig. 18 — Portion of germinating seed in longitudinal section (30 days). The fluorescence due to Fluoral yellow 88 staining shows the accentuated lipid decrease (70x).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cotyledon apex, while a discrete amount remains in the more chalazal portion. The protease test shows a reduction which is parallel to that of the protein reserves (fig. 16). Also the polysaccharide reserves have noticeably decreased (fig. 17), as have the lipid reserves (fig. 18), and calcium oxalate almost completely disappeared.

Transmission electron microscopy

Mature seeds

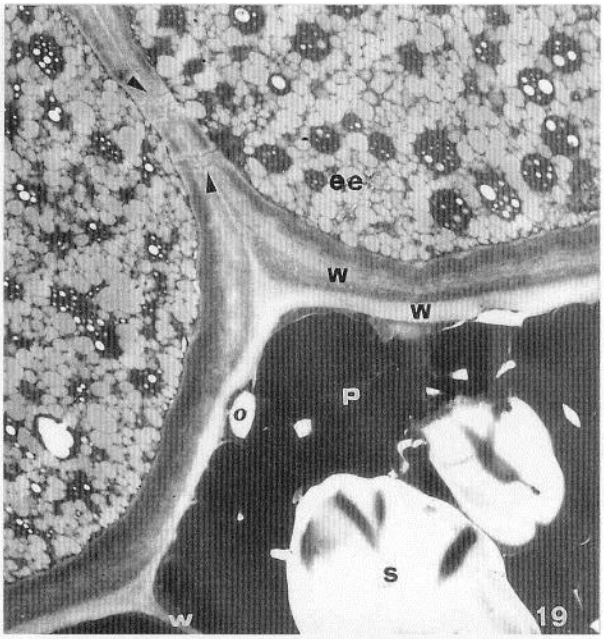
In the mature seed, the cells of the endosperm epidermis have anticlinal walls with numerous plasmodesmata and rather thick internal periclinal walls (fig. 19). In the cytoplasm the nucleus, lobed and showing condensed chromatin masses, is surrounded by mitochondria and proplastids (fig. 20). Most of the cytoplasm appears filled with spherosomes whose lipid nature is confirmed by the specific L.M. staining. These spherosomes surround aleurone granules provided with numerous small globoids; these for the most part are shattered and/or lost in the sectioning (figs. 19, 20). The presence of aleurone grains is confirmed by the specific L.M. staining for proteins. The endospermal parenchyma has cells with thin walls, without any plasmodesmata or intercellular spaces. These cells have no nucleus, plasmalemma or other organelles. They are completely filled with reserve substances: L.M. PAS positive starch granules and conspicuous clearly osmiophilic structures showing a polygonal outline; these structures are stacked one beside the other and exhibit a paracrystalline texture when observed at high magnification (figs. 19, 2la). The bodies respond positively to the specific L.M. staining for proteins. Among these paracrystalline inclusions some prismatic electrontransparent figures are present; they appear small and isolated or grouped together to create forms with very rugged edges (figs. 19, 22). These figures represent the sites filled prior to sectioning by the calcium oxalate crystals observed with L.M. Both the starch and these crystals are more abundant close to the cotyledon apex where, instead, the paracrystalline protein bodies tend to be fewer and smaller (fig. 22).

The cells of the cotyledon epidermis and parenchyma reveal thin walls and the absence of intercellular spaces. The ultrastructural characteristics of the cell contents are similar to those of the endosperm epidermis (figs. 22, 23). The specific L.M. stainings demonstrate that also the reserve substances of the above cells have the same nature.

Germinating seeds

7 days — The anticlinal and periclinal internal walls of the endosperm epidermal cells reveal a non homogeneous modification in the

Figs. 19-42: Transmission Electron Microscopy. Fig. 19 — Mature seed. Portion of endosperm: in the epidermis, the anticlinal walls have plasmodemata (arrow heads) and the internal periclinal walls are quite thick. The cells of the endosperm parenchyma have thin walls, many polygonal osmiophilic protein inclusions and starch granules. Holes of small single crystals shattered and/or lost during sectioning are visible (4,800x)Fig. 20 — Mature seed. Portion of an endosperm epidermal cell: the lobed nucleus is surrounded by proplastids and mitochondria (double arrows). The cytoplasm is rich in spherosomes and aleurone granules whose glo-boids are mostly shattered and/or lost during sectioning (5,800x). Fig. 21 — Mature seed. Portion of endosperm parenchyma cell rich in protein inclusions. Some holes of shattered and/or lost calcium oxalate crystals are present (21,300x). Fig. 21a — Detail of Fig. 19. The protein inclusions show a paracrystalline lattice arrangement (lll,400x). Fig. 22 — Mature seed. In the vicinity of the cotyledon apex stacked wall residues, abundant starch granules and holes of shattered and/or lost calcium oxalate crystals are seen. The cotyledon epidermal cells have thin walls and similar contents to those of the endosperm epidermis (3,300x). Fig. 23 — Mature seed. Cells of the cotyledon parenchyma: the walls are thin; intercellular spaces are absent; the cytoplasm contents are similar to those of the endosperm epidermis (7,300x).
Fig. 24 — Germinating seed (7 days). Portion of endosperm. The epidermis cell walls show a heterogeneous change in electron-density. In the parenchyma, the starch granules are reduced in size, the protein bodies appear unchanged (6,500x). Fig. 25 — Germinating seed (7 days). Portion of an endosperm epidermal cell: the cytoplasm is richer in proplastids and mitochondria (double arrows); several glyoxysomes can be identified; the matrix of the aleurone granules is mostly granular (8,300x). Fig. 26 — Germinating seed (7 days). Portion of endosperm: acid phosphatase (arrows) is present in aleurone grains, on the epidermis plasmalemma and at the level of the residual original plastidial envelope, in the endosperm parenchyma (7,100x). Fig. 27 — Germinating seed (7 days). Portion of endosperm parenchyma in the vicinity of the cotyledon apex: starch granules of reduced size, fragmented protein inclusions, and holes of shattered and/or lost calcium oxalate crystals can be seen (5,800x). Fig. 28 — Germinating seed (7 days). In the endosperm parenchyma just in the vicinity of the cotyledon apex a few starch granules reduced in size and holes of shattered and/or lost small calcium oxalate crystals remain (3,500x). Fig. 29 — Germinating seed (7 days). Portion of cotyledon epidermis: imaginations (arrows) of the highly osmiophilic plasmalemma work their way in between the abundant spherosomes and make contact with aleurone granules transforming into vacuoles (21,300x).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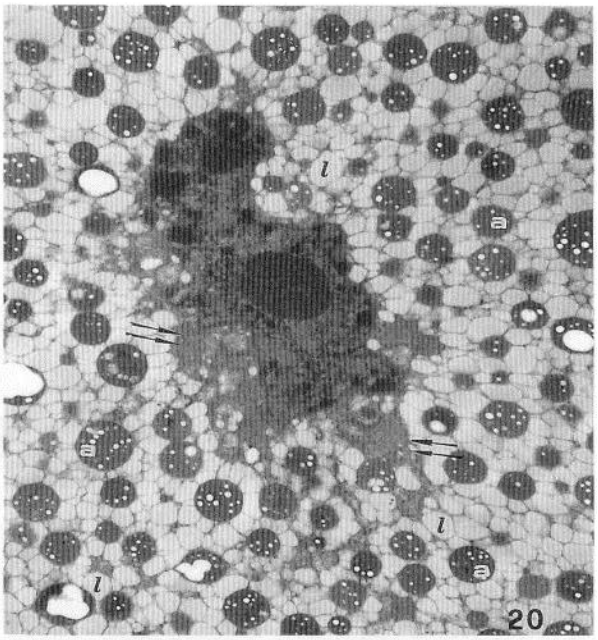
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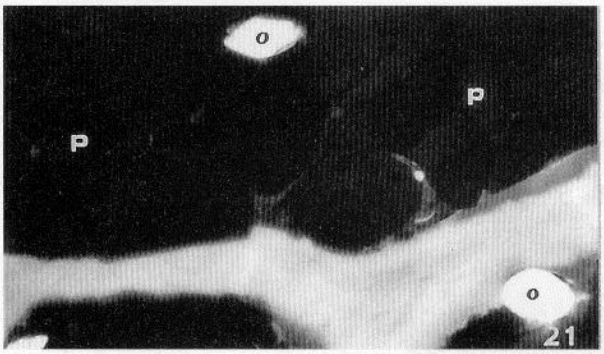
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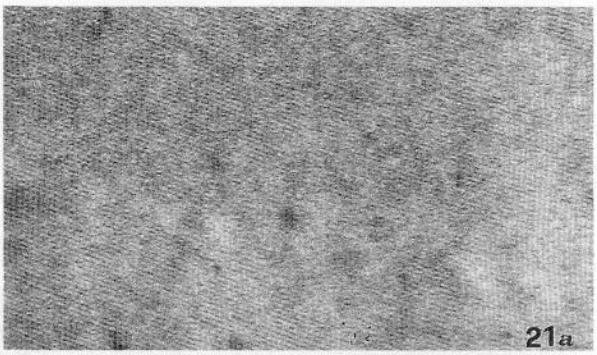
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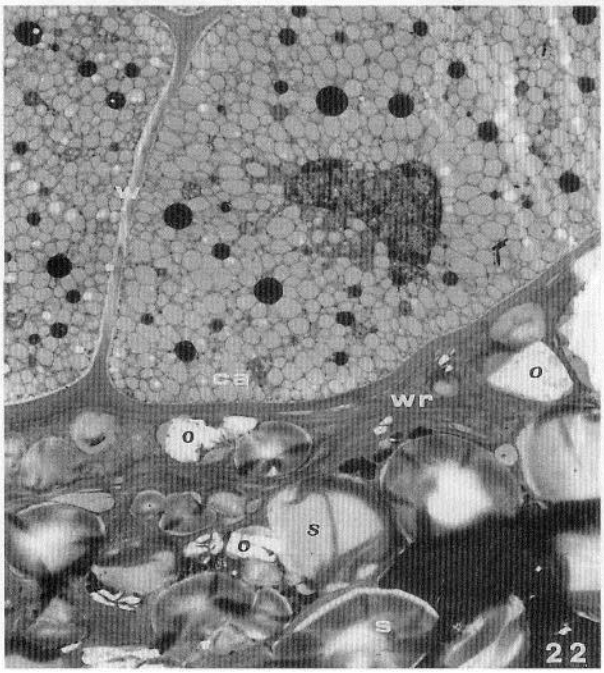
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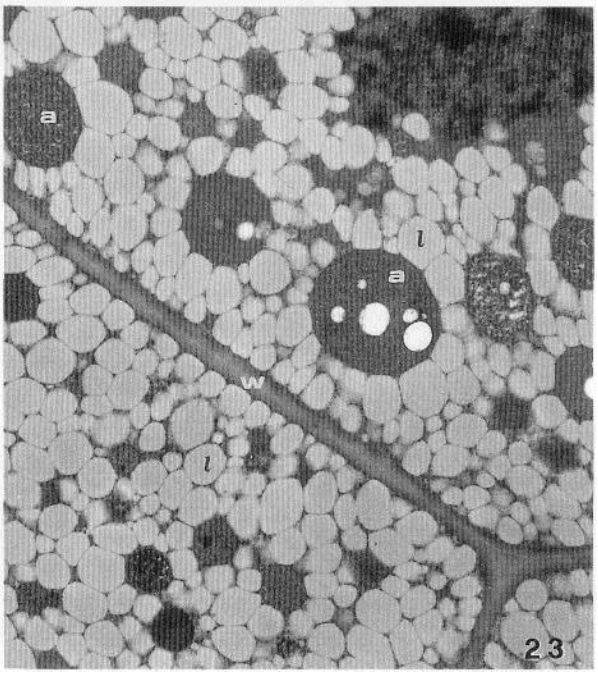
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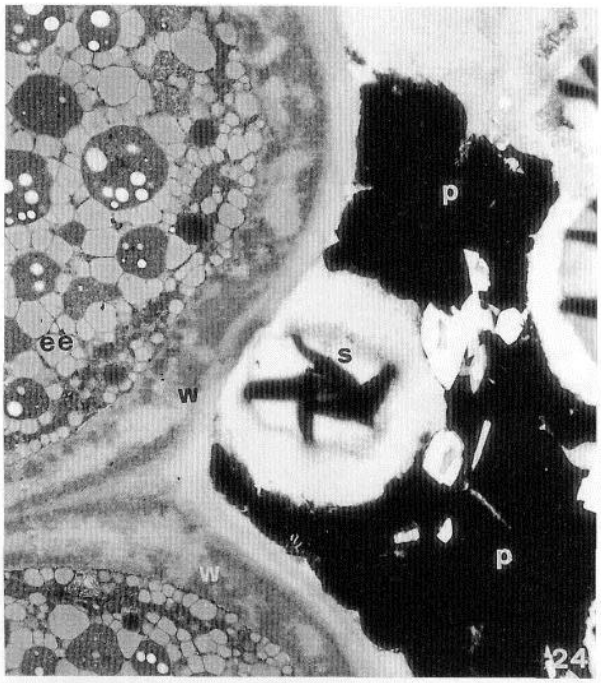
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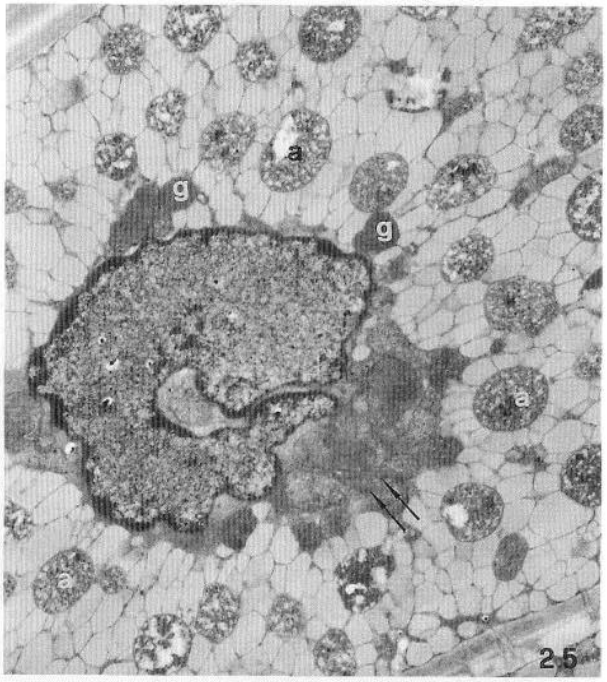
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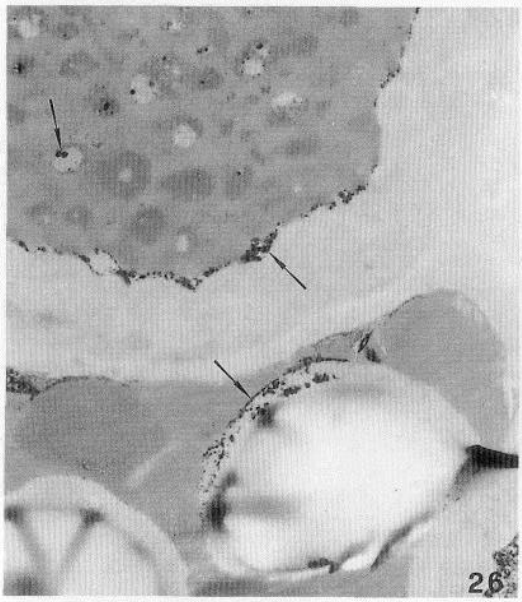
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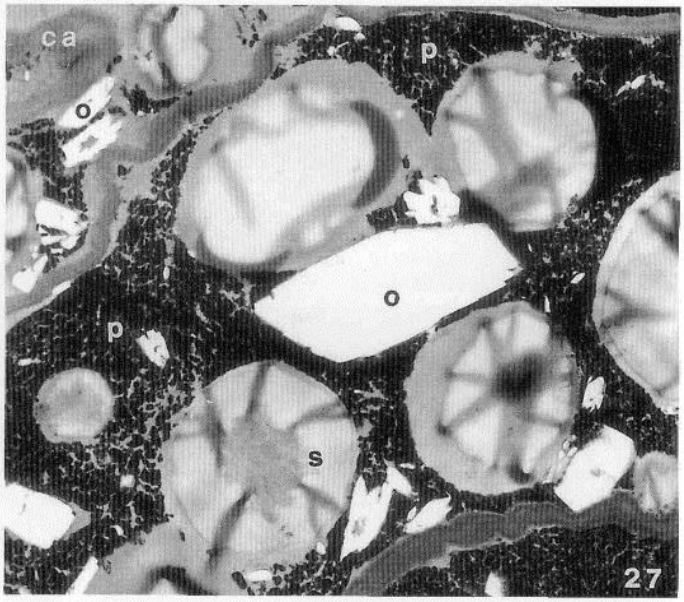
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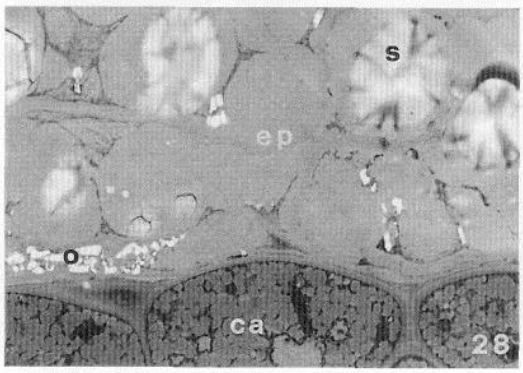
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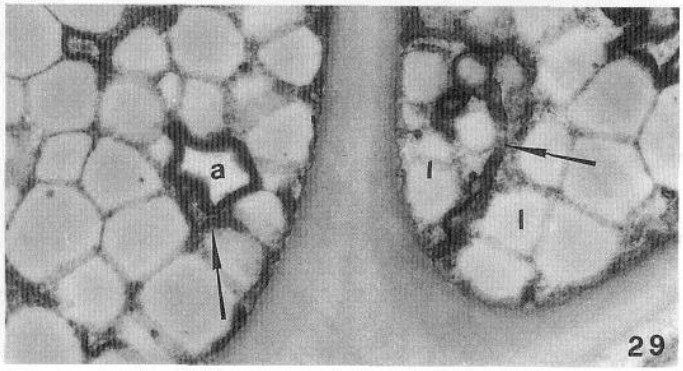
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electron-density (fig. 24). When compared to the mature seed, proplastids and mitochondria are more abundant in the cytoplasm, both in the perinuclear area as well as in the more peripheral region, scattered among the spherosomes. The protein matrix of many aleurone granules reveals a looser structure. Several microbodies are present which can be interpreted as glyoxysomes (figs. 24, 25). In the cells under examination, the specific reaction to highlight acid phosphatase gives a positive result at the level of the aleurone granules and of the plasmalemma (fig. 26). With reference to the endospermal parenchyma, a reduction in the starch granules size (figs. 24, 28) and the presence of acid phosphatase where the original plastid envelope was, can be observed (fig. 26). The paracrystal-line protein bodies appear fragmented as one moves from the chalazal region towards the cotyledon apex (fig. 27). In the proximity of the latter, they are completely absent (fig. 28).

In the cells of the cotyledon epidermis, the most noteworthy characteristic is the plasmalemma which is clearly osmiophilic; the plasmalemma also presents deep and narrow invaginations. The latter weave their way between the spherosomes and make contact with the aleurone granules which are in the process of transformation into vacuoles (fig. 29). These vacuoles present optically empty areas and osmiophilic remnants of the original contents; they are often irregular in shape (fig. 30). Plasmalemma and aleurone granules respond positively to the specific reaction to highlight the acid phosphatase. Either mitochondria or proplastids, or both, are present among the abundant spherosomes. In the cells of the cotyledon parenchyma, one can notice the presence of chloroplasts at different differentiation stages; these organelles show small starch granules. The aleurone granules have no globoids and show a loosely scattered, osmiophilic content (fig. 31). Glyoxysomes can be observed among the spherosomes (fig. 31).

15 days — The cells of the endosperm epidermis show a thinning out of all the walls and the appearance of plasmodesmata also in the internal periclinal walls. In the cytoplasm there is an increase in ribosomes and the development of endomembranes mainly as smooth endoplasmic reticulum; dictyosomes are also present. A spherosome decrease is evident. The aleurone granules are almost all transformed into vacuoles electron-transparent or containing more or less osmiophilic polymorph deposits; some vacuoles tend to fuse. The microbodies are more numerous: some show the glyoxysome typical core described in *Tillandsia* (BRIGHIGNA *et al.* 1982) (fig. 32). In the endosperm parenchyma the cell walls show frayed outlines. Among the walls some intercellular spaces appear (fig. 33). The starch granules present more reduced dimensions and often show a ring-like shape. A greater breakdown also effects the protein crystalloids which appear greatly fragmented also in the endosperm central portion. The calcium oxalate crystals are reduced in number and size (fig. 34).

In the neighbourhood of the cotyledon the endosperm parenchyma cells appear empty and the walls stacked beside each other; these constitute a more compact complex where they are in contact with the cotyledon (fig. 35). In the epidermal cells of the cotyledon apex, the tangential walls in front of the endospermal parenchyma show, on the external surface, electron-transparent material with a "foam-like" appearance. The internal contour of the same walls develops ingrowths lined by the plasmalemma. In the wall thickness, more electron-dense material is present; this is granular and fibrillar, and shows a decreasing concentration moving from the external towards the internal border of the wall (fig. 36). In the cytoplasm the aleurone granules are absent. The vacuolar apparatus is well-developed; its contents are finely granular and sometimes some more conspicuous osmiophilic precipitates are present (fig. 35). Numerous vesicles with barely electron-dense contents are scattered throughout the cytoplasm and frequently appear right next to vacuoles so much so as to determine invaginations of the tonoplast (fig. 36). A decrease in the spherosomes is observed and glyoxysomes appear. The cytoplasm is rich in ribosomes, mitochondria, chloroplasts and dictyosomes (figs. 35, 36). In the cotyledon parenchyma the appearance of small intercellular spaces, the absence of aleurone granules, the development of a conspicuous vacuolar apparatus and the appearance of some glyoxysomes represent the most noticeable changes (fig. 38).

30 days — The endosperm epidermal cells are characterized by a prominent vacuolar apparatus; the aleurone granules are absent, the spherosomes are greatly decreased in number

(fig. 39). The endosperm parenchymal cells show contents which are reduced to small residues of protein crystalloids (fig. 40). Also the complex of stacked walls close to the cotyledon is reduced.

The cotyledon epidermal cells appear lengthened tangentially. The vacuolar apparatus is well-developed and has mostly electron-transparent contents. In the scanty peripheral cytoplasm chloroplasts, mitochondria and ribosomes can be seen; a sharp reduction in spherosomes, glyoxysomes and endomembranes is clearly seen (fig. 41). In the cotyledon parenchyma cells the most evident changes are the presence of starch in the chloroplasts and the thickening of the tonoplast against which osmiophilic residues are stacked. Several spherosomes remain, whereas the glyoxysomes are less numerous (fig. 41).

DISCUSSION

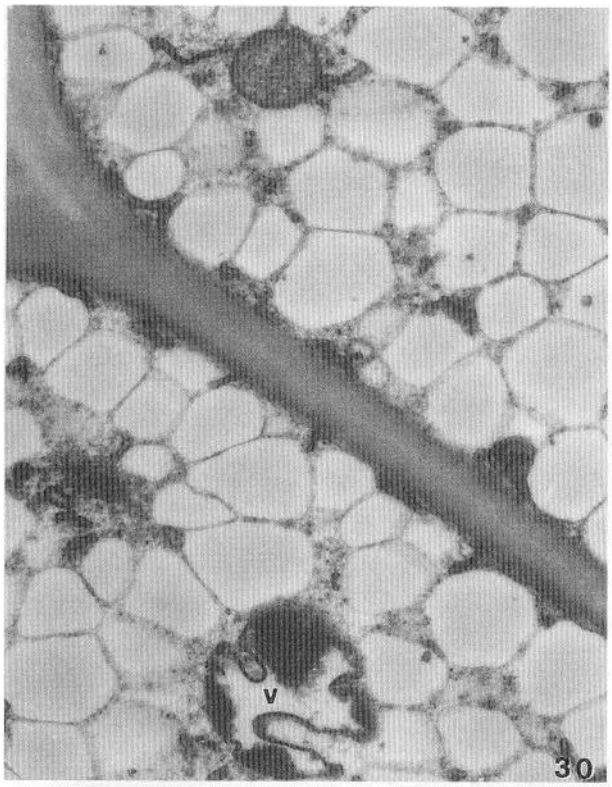
The results of L.M. and T.E.M. morphological observations, together with the cytochemi-cal reactions, indicate that the seed endosperm of *Tillandsia* accumulates the typical biological macromolecules, lipids, proteins, and polysaccharides, which are present in the seeds of the monocotyledons in different combinations. Nonetheless, their localization differs from what generally found in the monocotyledons with a well-developed endosperm; the known data are, for the most part, related to biochemical studies; from a morphological viewpoint, they concern only the endosperm of cereals (JACOBSEN 1984). Whereas in the latter, the endosperm usually represents 90% of the seed dry-weight and the embryo only 5% (JACOBSEN 1984), in *Tillandsia* the embryo, because of the conspicuous cotyledon, can account for as much as 25% of the dry-weight and the endosperm up to 65% (BENZING 1980).

In the cotyledon of *Tillandsia* there are reserves made up of soluble proteins and phytin gathered together in the aleurone granules scattered among the prevalent fats. It is obvious that an abundant presence of lipid material offers an advantage in the anemophylous dispersal of seeds which in compulsory epiphyte plants must be transported at a considerable height.

Reserves, analogous to the cotyledon ones, characterize the endosperm epidermis which thus constitutes an aleurone layer as happens in the Graminaceae. The localization of the remaining endosperm is different in *Tillandsia;* here it is placed mainly in front of the cotyledon apex and also fills the seed chalazal portion. Unlike the Graminaceae, the endosperm contents are not mostly polysaccharides, rather, they are made up, in almost equal parts, also of large protein crystals; moreover, there is a certain abundance of calcium oxalate crystals in the form of prisms or druses.

Fig. 30 — Germinating seed (7 days). Portion of cotyledon epidermis: the aleurone grain derived vacuoles present optically empty areas and highly osmiophilic residues of their original content (10,700x). Fig. 31 — Germinating seed (7 days). Portion of cotyledon parenchyma: besides the numerous spherosomes, chloroplasts at different differentiation stages with small starch granules can be seen as well as glyoxysomes (arrows), aleurone granules with loosely scattered osmiophilic contents (7,200x). Fig. 32 — Germinating seed (15 days). Portion of endosperm: in the cytoplasm of the epidermis cells a decrease in the number of spherosomes and the development of endomembranes, mitochondria, glyoxysomes and ribosomes can be noted. Large aleurone grain derived vacuoles have a polymorph content. The parenchyma shows highly fragmented protein crystals (15,000x). Fig. 33 — Germinating seed (15 days). Portion of endosperm parenchyma: note the degradation and separation of the cell walls; fragmented protein crystal residues are present (8,500x). Fig. 34 — Germinating seed (15 days). Portion of endosperm parenchyma: protein crystals and starch granules in stages of increasing degradation towards the cotyledon apex can be seen; few and small holes of shattered and/or lost calcium oxalate crystals are present. (3,600x).'

Fig. 35 — Germinating seed (15 days). Portion of cotyledon and of endosperm parenchyma: the cells of the latter are empty and the wall residues very close to the cotyledon are heavily stacked together. In the cotyledon epidermis, the vacuoles are well developed and have a polymorph content; vesicles with a mostly electron-transparent content, chloroplasts, ribosomes, mitochondria (arrows) and few spherosomes are present (3,600x). Fig. 36 — Germinating seed (15 days). External tangential wall of a cotyledon apex epidermis cell: the external surface is associated with electron-transparent material characterized by a "foam-like" morphology; the internal wall surface develops ingrowths (arrow heads) lined by the plasmalemma; more electron-dense fibrillar and granular material (arrows) is present in the parietal matrix (41,600x). Fig. 37 — Germinating seed (15 days). Portion of cotyledon epidermis: some of the many vesicles with barely electron-dense content push the tonoplast into the vacuole; the internal surface of the external tangential wall shows minute ingrowths (arrow heads) lined by the plasmalemma (9,850x). Fig. 38 — Germinating seed (15 days). Portion of cotyledon parenchyma: intercellular spaces appear; the cytoplasm presents conspicuous vacuoles, chloroplasts, spherosomes and some glyoxysomes (3,600x).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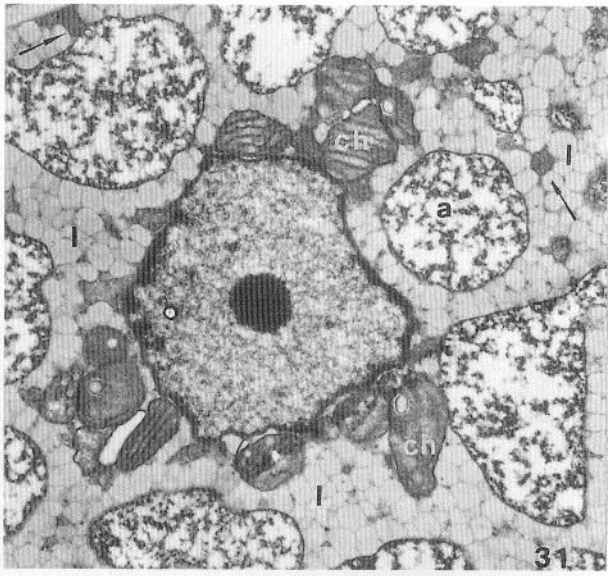
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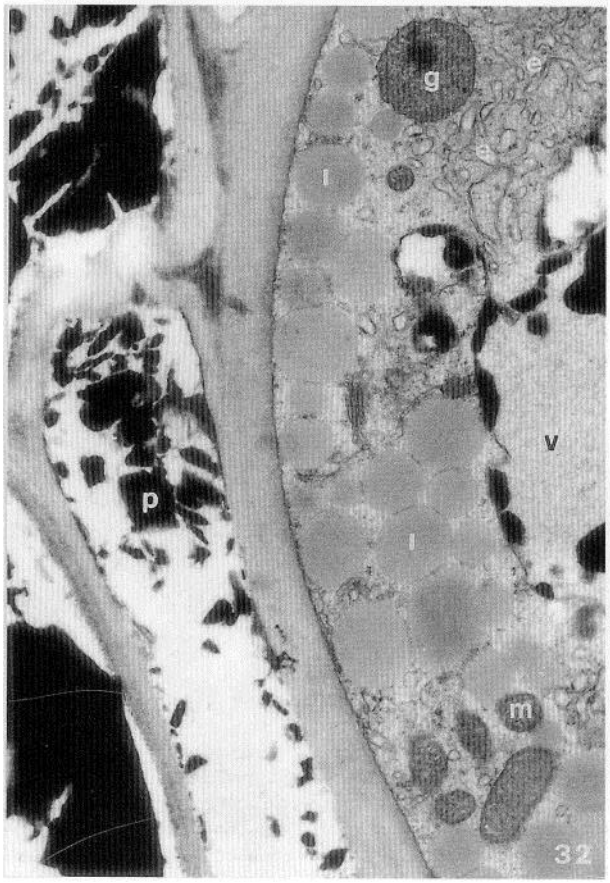
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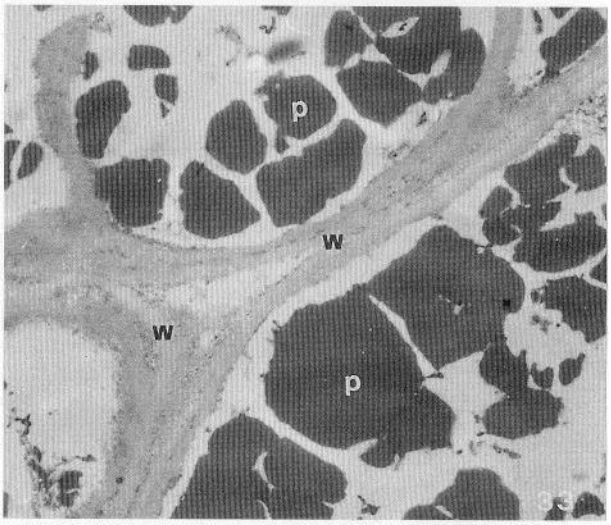
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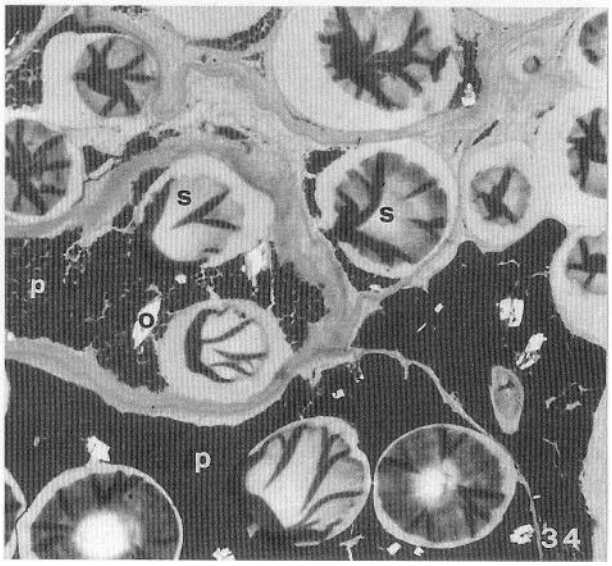
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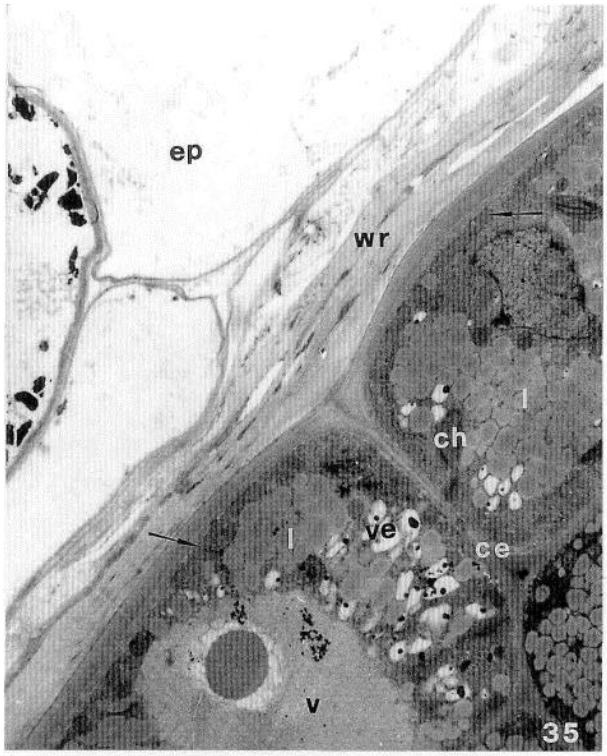
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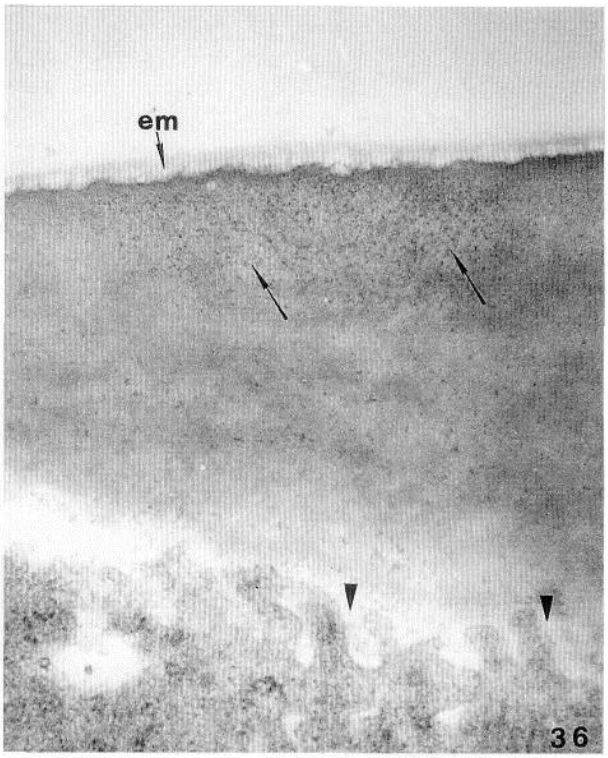
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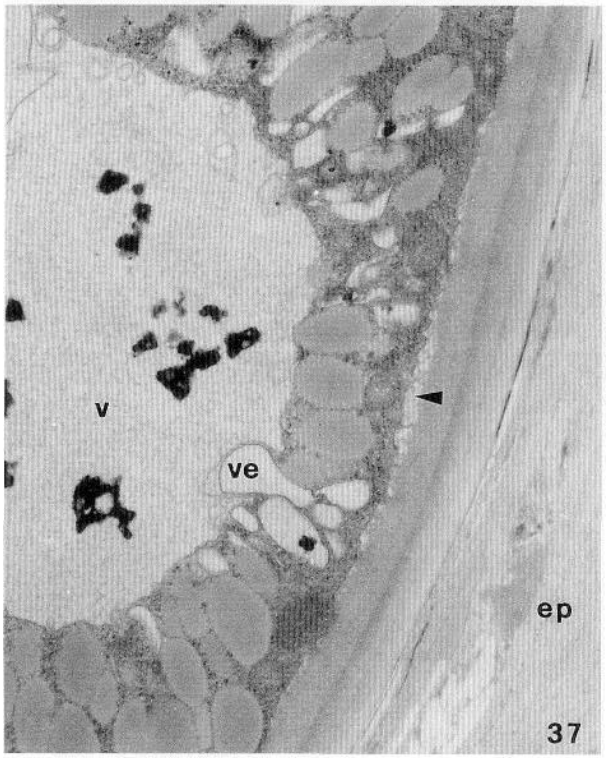
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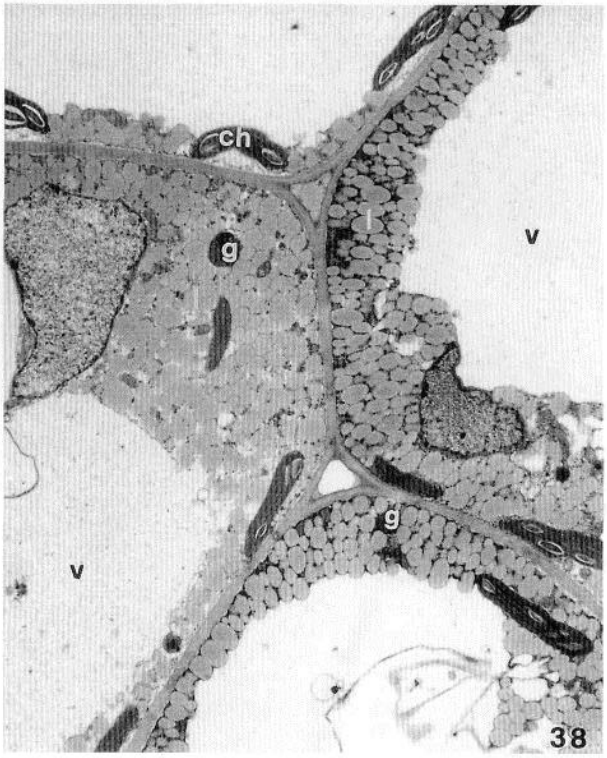
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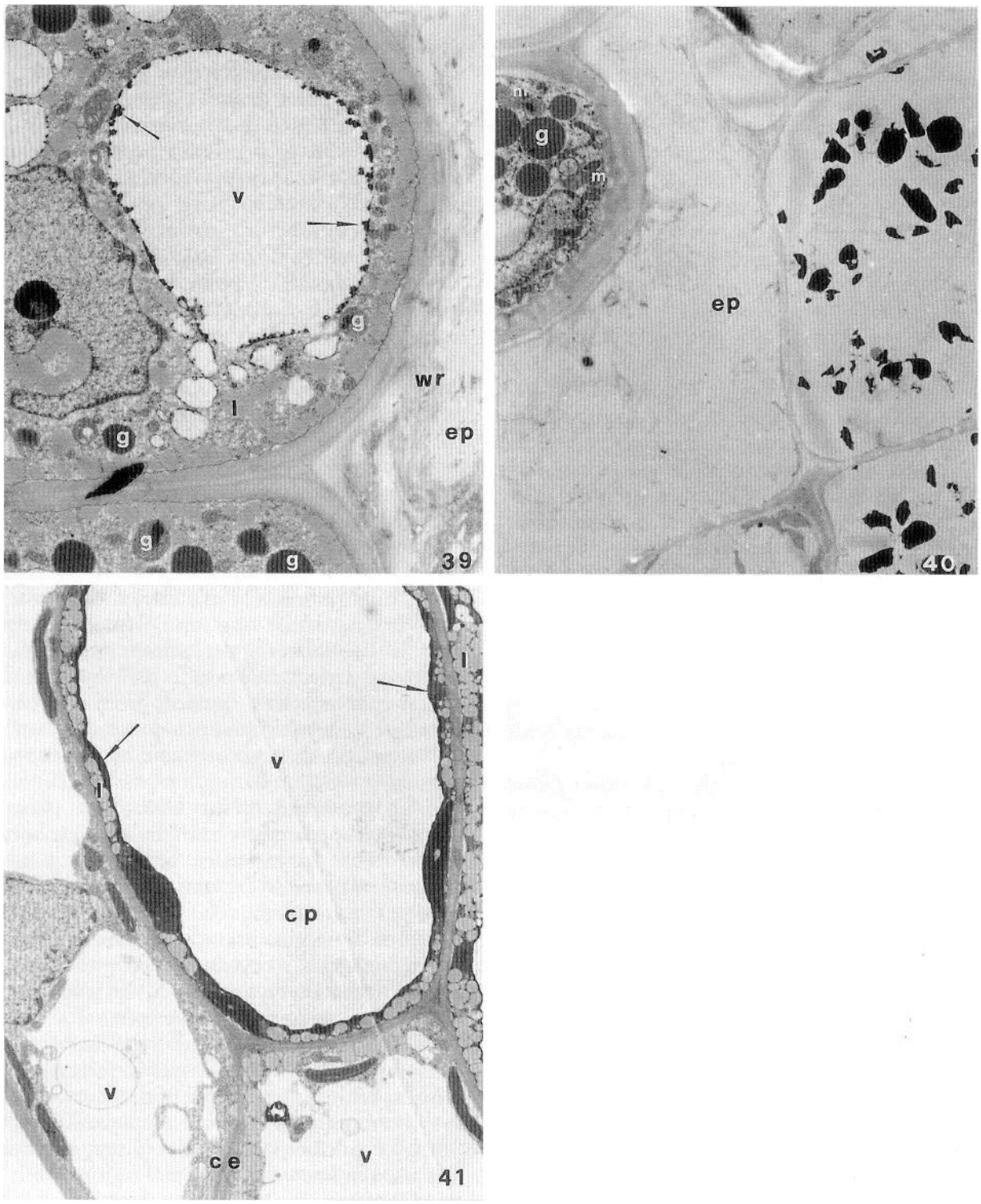

Fig. 39 — Germinating seed (30 days). Portion of endosperm: the disappearance or the aleurone grains can be noticed, together with the reduction of the spherosomes, the increase in the glyoxysomes and the development of vacuoles with peripheral osmi-ophilic residues (arrows). The adjacent endosperm parenchyma is made up of parietal residues (3,600x). Fig. 40 — Germinating seed (30 days). Portion of endosperm: the abundance of glyoxysomes and mitochondria in the epidermis is quite obvious. The reduction in all the reserves is evident in the parenchyma (3,600x). Fig. 41 — Germinating seed (30 days). Portion of cotyledon: in the epidermis, the vacuoles are well developed; in the scarce cytoplasm, chloroplasts, mitochondria and ribosomes prevail. In the parenchyma, the tonoplast of the sole large vacuole is thickened by osmiophilic deposits (arrows); the chloroplasts present some small starch granules; various spherosomes still remain (3,600x).

Another new datum, compared to what previously known about the seeds of other monocotyledons, is the presence in the *Tillandsia* seed of a complex of stacked walls which almost completely surrounds the embryo. These wall layers, most probably cellulose wall residues of endosperm cells, indicate that the endosperm plays a role also in the development of the embryo. This is in accordance with the hypothesis proposed by SARFATTI (1964), MASAND and KAPIL (1966), BOESEVINKEL and BOUMAN (1984), rather than with the concept of endosperm as a reserve tissue exclusively involved in the germination of the seed (VIJAYARAGHAVAN and PRAB-HACAR 1984). Moreover, in the case of *Tillandsia*, the fact of maintaining parietal polymers in the seed which, like cellulose, cannot be broken down by an enzymatic complement in situ, can favour the setting up of an association with het-erotroph organisms. The latter, through their metabolism, could offer a useful trophic contribution for the early development phase of the seedling in extreme environments, such as those which characterize the ecology of these plants. Indeed, FRANCINI CORTI (1981) hypothesized for *Tillandsia* an association with heterotroph micro-organisms fit for breaking down residues inside the plant itself so as to provide nutrients useful for the seed formation.

On seed germination, the imbibition allows the use of the reserves to begin. According to what JACOBSEN (1984) observed in cereals in general, and what MITSUHASHI and OAKS (1996) observed in *Zea mays*, the activation of the hydrolytic enzymes pre-existing in the aleurone granules of the endosperm epidermis occurs and, thus, the synthesis of new hydrolytic enzymes. The latter are transported into the endosperm parenchyma as observed by BOTTARI *et al.* (1996) and by MOUSSAVI-NIK *et al.* (1998) in wheat.

In *Tillandsia*, as shown by the cytochemical reactions and by the ultrastructural changes, the transformation of the aleurone granules into vacuoles is observed, not only in the endosperm epidermis, but also in the epidermis and in the parenchyma of the cotyledon. A passage of material from the aleurone layer to the endosperm parenchyma is confirmed by the heterogeneous ultrastructural feature assumed by the internal tangential cell walls of the aleurone layer as well as by the anticlinal ones, by the appearance of plasmodesmata in the internal tangential walls and by the positive response of the internal tangential plasmalemma to the acid phophatase reaction. It is to be presumed that the aleurone layer in *Tillandsia* provides a greater quantity of hydrolytic enzymes than that released by the cotyledon. This fact has also been found for barley by RANKI and SAPONEN (1984) and for cereals in general by AKAZAWA and HARA-NISHIMURA (1985).

As known for the Graminaceae, also in *Tillandsia* the endosperm parenchyma, diversely to the aleurone layer, is made up of nonliving cells. From the present study it is found that in *Tittandsia* the enzymatic breakdown of the reserves is differentiated: the calcium oxalate crystals remain unchanged for a long period of time; the breakdown of the protein crystalloids is earlier in the neighbourhood of the cotyledon apex compared to the more chalazal endosperm; the reduction in the starch granules is instead simultaneous throughout the endosperm. With reference to the breakdown of the single granules, this starts on their outer part. The particular ring form assumed by the granules themselves in the sections indicates an additional activity by specific pre-existing and re-activated enzymes. Indeed, from the biochemical analyses conducted by JACOBSEN (1984) on cereals in general, and by RANKI and SAPONEN (1984) on barley, the presence of preexisting /3-amylase in the endosperm parenchyma and of a-amylase from the aleurone layer results. The pre-existence of amylolytic enzymes in *Tillandsia* explains the fact that the starch granules are the sole endosperm reserves bordered by a membrane, the remains of the original plastidial envelope. The presence of acid phosphatase on this membrane is to be related to intense transport processes.

With the progress of the germination, the consumption of the endosperm reserves increases. With the emergence of the first leaflets, the consumption is almost complete. The deposits of calcium oxalate also disappear; this fact was not known until now (LOTT 1980). Instead thin and diaphanous remnants of the cell walls remain. Also this aspect, which in *Tillandsia* takes on the above-mentioned special ecological meaning, differentiates the seed under examination from the seed of other monocotyledons studied: in the cereals, during germination, the first structures which are broken down are the cell walls of the endosperm parenchyma,

which are largely not cellulosic (JACOBSEN 1984); in *Yucca,* the hydrolysis of the cell walls, highlighted by formation of small localised "pockets", is earlier than that of the starch and lipids (HORNER and ARNOTT 1965, 1966).

The intense metabolic activity correlated with the germination process also involves the use of the embryo spherosomes and those of the endosperm epidermis, mediated by abundant glyoxysomes. In agreement with CHRISPEELS (1986), the development of the smooth endoplasmic reticulum in the same tissues is involved with the transport of the sugars. These sugars, derived from lipids, move from the endosperm epidermis towards the peri-embryonal space and from the embryo epidermis inside the embryo itself.

In the cells of the embryo epidermis, the early thickening of the plasmalemma, the subsequent increase in the wall-membrane apparatus surface and the appearance of material on the external surface of the external tangential walls indicate an intense and rapid absorption activ ity. All these features confirm the haustorial role of the cotyledon proposed for *Tillandsia* by FRANCINI (1981). The substances absorbed are very likely hydrolyzed endosperm reserves. These substances are destined in part to a temporary accumulation in the vacuolar apparatus, as indicated by the connections of some plasmalemma invaginations with the tonoplast. The immediate role of the nutrients accumulated is the uptake of water for growth distension; a swelling of the hypocotyl is clearly evident, so much so as to cause the rupture of the internal tegument. A contribution to the water uptake and to the normal embryo trophism can be assured also by the photosynthetic activity of the chloroplasts which develop abundantly in the cotyledon parenchyma. Thus, the absorption role of the embryonal radicle, lost in *Tillandsia* at an early stage (CECCHI FIORDI *et al.* 1996), is substituted, as is the role of the adventitious root apparatus; in the atmospheric species, the latter ends up having simply an anchoring role (MEZ 1904; BENZING 1980; BRIGHIGNA *et al.* 1990).

The low capacity to absorb water and mineral salts, including nitrates, from the environment continues until the development of absorbing foliar trichomes characteristic of *Tillandsia*. This fact also justifies the prevalent presence of protein reserves, aleuronic globoids, and calcium oxalate in the seeds. The storage of abundant nutrients and water in the cotyledon, which characteristically in *Tillandsia* is large, permits a quicker use of the nutrients themselves at germination. This is a further aspect which offers greater chances of success to the seedling.

REFERENCES

AKAZAWA T. and HARA-NISHIMURA I., 1985 — *Topographic aspects of biosynthesis, extracellular secretion and intracellular storage of proteins in plant cells.* Ann. Rev. Phychol., 36: 441-472.

BECCARI N. e MAZZI V., 1966 — *Manuale di tecnica microscopica.* Societa Editrice Libraria.

BENZING D.H., 1980 — *The biology of the Bromeliads.* Mad River Press, Eureka, California.

BOESEWINKEL F.D. and BOUMAN F., 1984 — *The Seed: Structure.* In: B. M. Johri (Ed.), "Embryology of Angiosperms", p. 567-610. Springer-Verlag, Berlin.

BOTTARI A., CAPOCCHI A., FONTANINI D. and GALLESCHI L., 1996 — *Major proteinase hydrolising Gliadin during wheat germination.* Phytochemis-try, 43: 39-44.

BRIGHIGNA L., CECCHI FIORDI A. and PALANDRI M.R., 1982 — *Observations on the microbodies in the Genus Tillandsia (Bromeliaceae).* Am. J. Bot.,69: 1179-1184.

—, 1990 — *Structural comparison between free and anchored roots in Tillandsia (Bromeliaceae) Species.* Caryologia, 43: 27-42.

BRUNDRETT M.C., KENDRICK B.- and PETERSON C.A., 1991 — *Efficient Lipid Staining in Plant Material with Sudan Red 7B or Fluoral Yellow 088 in Polyethylene Glycol-Glycerol.* Biotechnic and Histochemistry, p. 111-116.

BUTTROSE M.S. and LOTT J.N.A., 1978 — *Calcium oxalate druse crystals and other inclusions in seed protein bodies Eucalyptus and Jojoba.* Can. J. Bot, 56: 2083-2091.

CECCHI FIORDI A., PALANDRI M.R., Di FALCO P. and TANI G., 1996 — *Cytological aspects of the hypocotyl correlated to the behavior of the embryo radicle of Tillandsia atmospheric species,* Caryologia, 49: 113-124.

CHRISPEELS M. J., 1980 — *The endoplasmic reticulum.* In: A. K. Stumpf and E.E. Conn (Eds.), "The Biochemistry of Plants", Vol. I, p. 390-412. Academic Press, New York.

EVERSON PEARSE A.G., 1968 — *Histochemistry Theoretical and Applied.* Vol. I. Little, Brown and Company, Boston.

—, 1972 —*Histochemistry Theoretical and Applied.* Vol. II. Churchill Livingstone, Edinburgh and London.

FRANCINI CORTI E., 1981 — *Epifitismo e sua evoluzi-one.* — In: VII Seminario sull'evoluzione biologica e i grandi problemi della biologia — Atti Ace. Naz. Lincei, Roma.

HORNER H.T. and ARNOTT H.J., 1965 — *A his tochemical and ultmstructural study of Yucca seed proteins.* Am. J. Bot., 52: 1027-1038.

—, 1966 — *Histochemical and ultrastructural study of pre- and post-germinated Yucca seeds.* Bot. Gaz., 127: 48-64.

JACOBSEN J.V., 1984 — *The Seed: Germination.* In: B. M. Johri (Ed.), "Embryology of Angiosperms", p. 611-646. Springer-Verlag, Berlin.

JENSEN W.A., 1962 — *Botanical histochemistry.* W.H. Freeman and Company, San Francisco and London.

LOTT J. N. A., 1980 — *Protein bodies.* — In: P. K. Stumpf and E. E.Conn (Eds.), "The Biochemistry of Plants", Vol. I, p. 589-623. Academic Press, New York.

MAS AND P. and KAPIL R. N., 1966 — *Nutrition of the embryo sac and embryo. — A morphological approach.* Phytomorphology, 16: 158-175.

MEZ C., 1904 — *PhysiologischeBromeliaceen — Studien. I, Die Wasserokonomie der extrem atmospha'rischen Tillandsien.* Jahrb. Wiss. Bot., 40: 157-229.

MITSUHASHI W. and OAKS A., 1996 — *Localization of major endopeptidase activities in maize endosperm.].* Exp. Bot., 47: 749-754.

MoUSSAVI-NIK M., PEARSON J.N., HOLLAMBY GJ. and GRAHAM R.D., 1998 — *Dynamics of nutrient remobilization during germination and early seedling development in Wheat.* J. Plant Nutr., 21: 421-434.

O'BRIEN T.P. and McCULLY M.E., 1981 — *The study of plant structure. Principles and selected methods.* Termarcarphi Pty. Ltd., Melbourne.

Poux N., 1970 — *Localization d'activites enzimatiques dans le meristeme radiculaire de Cucumis sativus L. IE: Activite Phosphatasique Acide.* J. Microsc., 9: 407-434.

RANKI H. and SAPONEN T., 1984 — *Secretion of a-amilase by the aleurone layer and the scutellum of germinating barley grain.* Plant Physiol., 75: 710-715.

REYNOLDS E.S., 1963 — *The use of lead cytrate at high pH as an electron opaque stain in electron microscopy.* J. Cell. Biol., 17: 208-212.

SARFATTI G., 1964 — *Le basi anatomiche dei rapporti nutritivi tra pianta madre, seme ed embrione.* Giorn. Bot. Ital., 71: 345-348.

SPURR A.R., 1969 —*A low-viscosity epoxy resin embedding medium for electron microscopy.* J. Ultrastr. Res., 26: 31-43.

TWINING S. S., 1984 — *Fluorescein Isothiocyanate-labeled Casein Assay for Proteolytic Enzymes.* An. Biochem., 143:30-34.

VIJAYARAGHAVAN M.R. and PRABHAKAR K., 1984 — *The Endosperm.* In: B.M. Johri (Ed.), "Embryology of Angiosperms", p. 319-376. Springer-Verlag, Berlin.

WATSON M.L., 1958 — *Staining of tissue sections for electron microscopy with heavy metals.* J. Biophys. Biochem. Cytol., 4: 475-478.

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