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# DEVELOPMENT OF OVULE AND TESTA IN RUTACEAE III. SOME REPRESENTATIVES OF THE AURANTIOIDEAE

F. D. BOESEWINKEL

Hugo de Vries-laboratorium, Universiteit van Amsterdam

SUMMARY

The ovule primordia of *Poncirus* and *Citrus* are trizonate and the outer integument is of subdermal origin. The seed-coats of *Poncirus, Citrus, Fortunella, Citropsis* and *Murraya* are very similar in structure but differ in minor points. The mechanical layer is a derivative of the outer epidermis of the outer integument. In the five taxa studied the seed coat is formed out of the outer integument and usually also contains remains of the inner integument and the nucellus. The innermost layers of the outer integument often contain crystals. A thin layer of endosperm one to a few cell layers thick is present.

## 1. INTRODUCTION

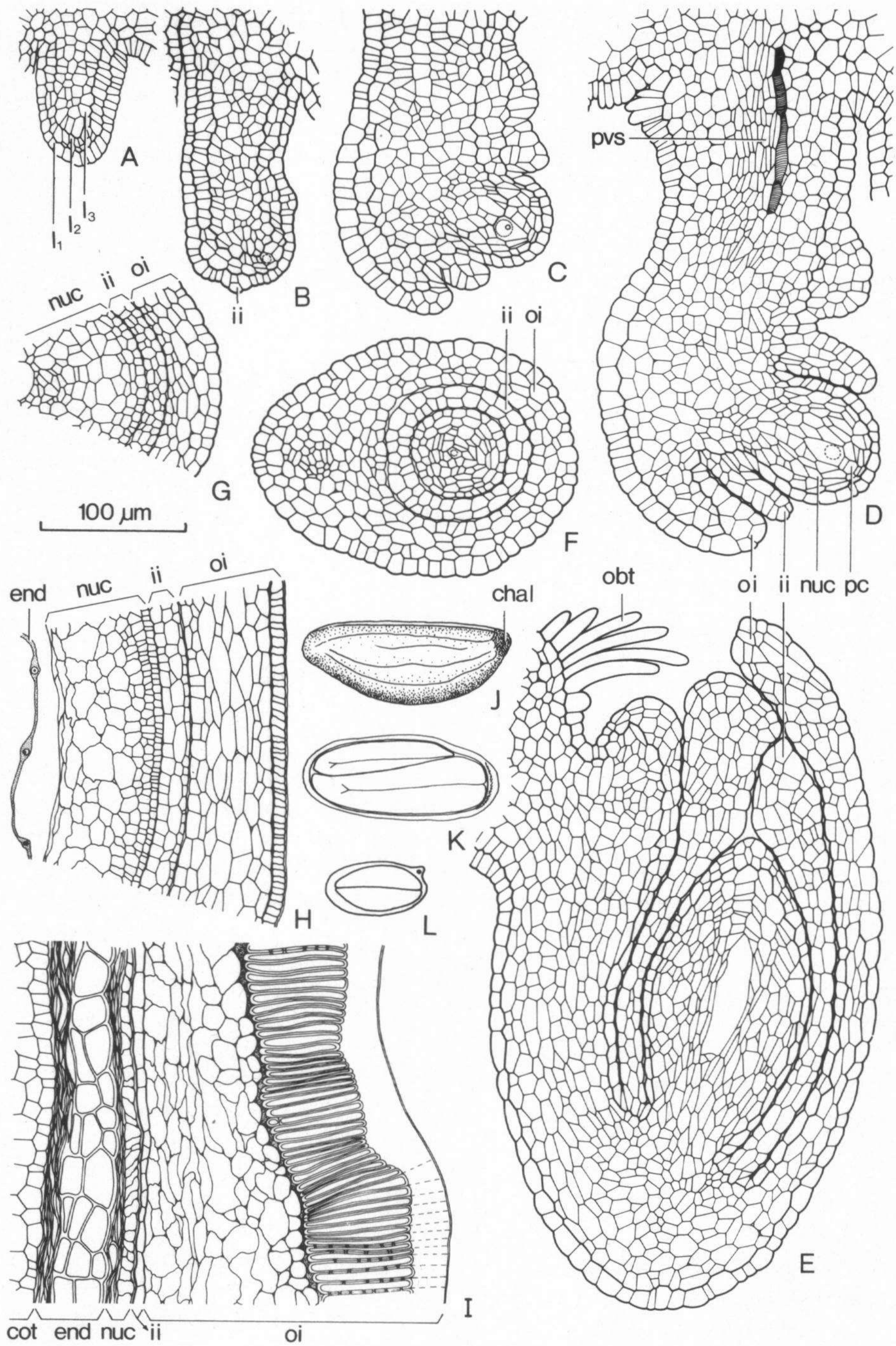
The representatives of the Aurantioideae are all evergreen trees or shrubs, with the exception of the three deciduous and monotypic genera *Poncirus, Aegle* and *Feronia.*

The ovules of the Rutaceae are according to NETOLITZKY (1926) and DAVIS (1966), see also BOESEWINKEL (1977), bitegmic, crassinucellate, and anatropous. Only within the genus *Glycosmis* of the Aurantioideae do unitegmy and pachychalazy occur (BOESEWINKEL & BOUMAN 1978). The ovules and seeds of the following bitegmic representatives of the Aurantioïdeae have been studied till now: *Citrus* (BIERMANN 1897; GALLET 1913; BANERJI 1954; CORNER 1976), *Murraya, Triphasia* and *Atalantia* GALLET 1913: CORNER 1976), *Feronia* = *Limonia* (GALLET 1913; BANERJI & PAL 1958; CORNER 1976), and *Aegle* GALLET 1913; JOHRI & AHUJA 1957).

A detailed description of the ontogeny of the ovule in the Aurantioideae had not previously been given. According to GALLET (1913), the Aurantioideae constitute the only subfamily of the Rutaceae in which the genera show a certain uniformity in the structure of their seed-coat.

## 2. MATERIAL AND METHODS

The material of *Poncirus trifoliata* (L.) Raf., *Citrus aurantium* L., and *Murraya paniculata* (L.) Jack. was collected from specimens cultivated in the Hortus Botanicus, University of Amsterdam. *Citropsis* cf. *C. tanakae* Swingle & M. Kell material was obtained from the hothouses of the Laboratory of Phytotaxonomy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and Phytogeography, Wageningen. Seeds of *Fortunella margarita* )Lour.) Swingle were obtained from fruits bought in a local market in Amsterdam.

Fixation was done in Craf and Allan-Bouin mixtures. Sections were made by means of standard microtome techniques and by hand. The following stains were used for specific colour tests: phloroglucinol and sulphuric acid, Sudan IV, ruthenium red and JKJ.

In order to remove the mucilaginous layer or "pellicle" of the seeds of *Poncirus, Citrus, and Citropsis*, the seeds were treated in a 10 per cent. solution of $HNO_3$. If the slimy layer was not completely removed by this treatment the seeds were placed in 30 per cent. $H_2O_2$ and left in this reagent for about an hour after which $KMnO_4$ was added during gentle heating.

The SEM photographs were made by means of a Cambridge Stereoscan Mark 2a.

## 3. RESULTS

### 3.1. Poncirus trifoliata

This species has the baccate type of fruit characteristic of the Aurantioideae. The endocarp is copiously developed as in *Citrus*. The internally 6-parted fruit contains many seeds.

*Ovule ontogeny*

The ovule primordium is trizonate; its initiation takes place by periclinal divisions in the corpus ($l_3$) surrounded by the initially only anticlinally dividing cell layers of the subdermatogen ($l_2$) and the dermatogen ($l_1$).

*Fig. 1A* shows a trizonate ovule primordium with beginning differentiation of the nucellus, as indicated by the periclinal divisions in the subdermal layer near the apex of the primordium. The initiation of the inner integument (i.i.) is dermal and comes about by the formation of periclinal and oblique walls in about three dermal cells (*fig. 1B, C*). In this way a circumvallation is formed. The i.i. remains mainly two cell layers thick except at the base where it is thicker and consists of more cell layers. When the ovule is about full-grown the cells of the innermost layer of the i.i. divide periclinally rendering the integument 3-layered (*fig. 1G*); this three-layered condition persists except in the micropylar part where it becomes multi-layered (*fig. 1E*). The initiation of the outer integument (o.i.) takes place in the subdermal layer: in *fig. 1B* subdermal cell divisions can be observed even before a bulge is

Fig. 1. Development of ovule and testa of *Poncirus trifoliata*. A, B, C, D, and E: l.s. of developing ovules, successive stages; F: tr.s. of developing ovule;
H and I: development of seed-coat as seen in tr.s. of the seed;
J: mature seed;
K and L: l.s. and tr.s. of ripe seed.
$l_1$, $l_2$ and $l_3$: dermal layer, subdermal layer and corpus, respectively.
end = endosperm; nuc. = nucellus; p.c. = parietal cells; ii = inner integument; o.i. = outer integument; pvs = provascular strand; obt = obturator; cot = cotyledon.

formed. Soon after this subdermal mitotic activity about three of the overlying dermal cells start dividing by periclinal and oblique walls (*fig. 1C*). The dermal cells are pushed up by the subdermal tissue. Soon after its initiation the o.i. attains a width of 4 to 5 cell layers (*fig. 1D*) but during the subsequent development it does not become any thicker. The o.i. forms an asymmetrical circular wall because it is clearly arrested in its development at the concave side and is there largely of dermal origin.

*The full-grown ovule*

The full-grown ovule is bitegmic, anatropous, and crassinucellate (*fig. 1E, G*). The nucellus is very large and has a conspicuous cap. The i.i. is three cell layers thick except in the distal parts. A clear dividing line is discernible, all along the length of the i.i., between the cells derived from the original inner layer and those derived from the outer layer. The o.i. is four to five cells in thickness except at the distal side, and longer than the i.i., but the micropyle is formed by both. In cross section endostome and endostome are star- to slit-shaped; the exostome is situated somewhat closer to the funicle. Starch is present throughout the ovule, but is especially copious in the embryo sac (e.s.) and in the apical portions of nucellus, i.i., and o.i. The proliferation of dermal cells of parts of the placenta and the funicle leads to the formation of an obturator chiefly consisting of unicellular hairs but containing some which became two-celled by a transverse division. The obturator contains but little starch or none at all.

*The development of the seed-coat*

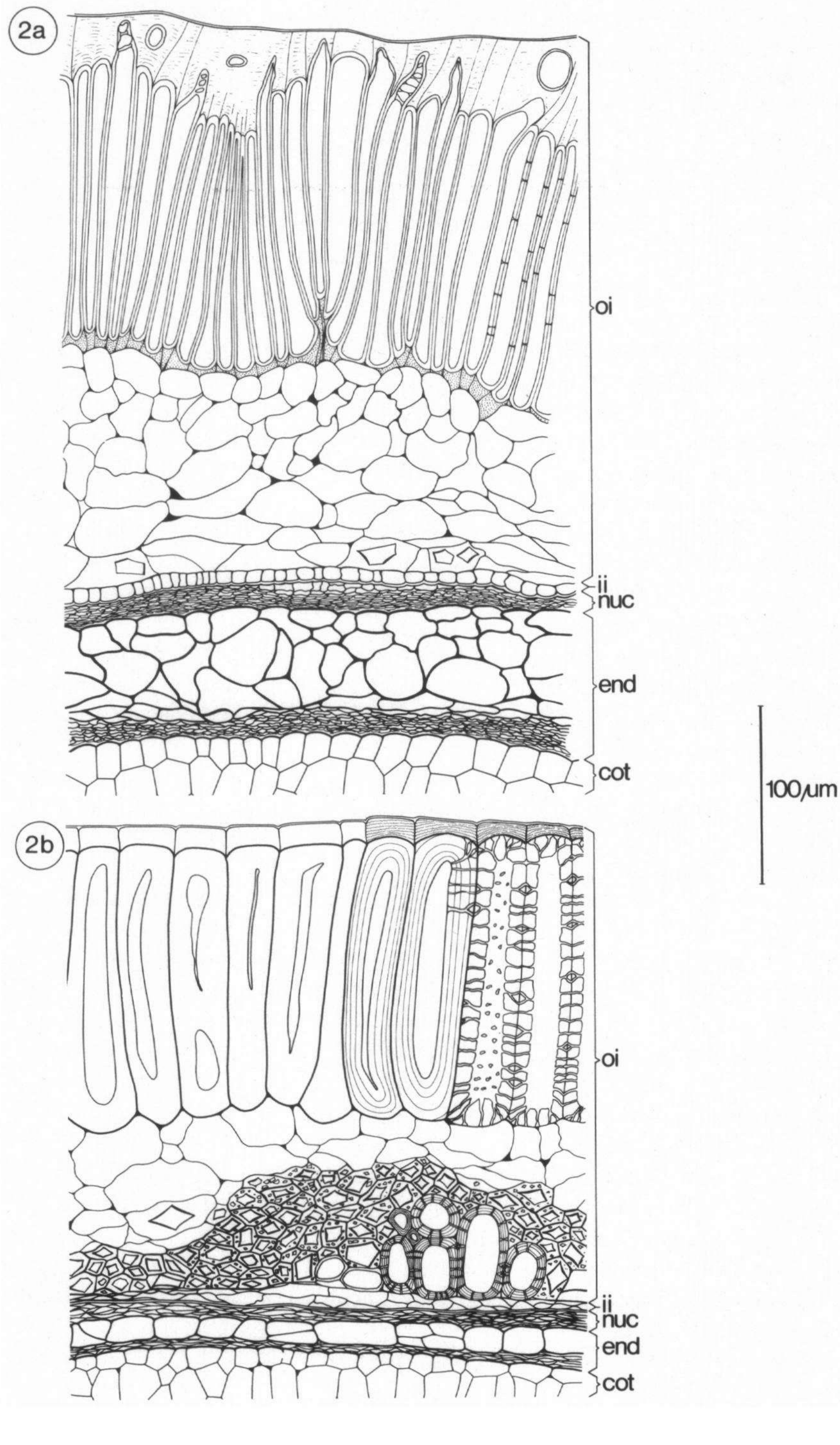
The testa differentiates during its development into various layers (*fig. 1H*). Especially the cells of the innermost and adjacent layers of nucellus, i.i., and o.i. enlarge, the nucellus becoming very large after fertilisation. Whilst the nucellus is internally being resorbed by the strongly extending e.s., it continues to grow by periclinal divisions taking place especially in the peripheral layers. The cells of the innermost layer of the i.i. are rich in cytoplasm; its outer two cell layers are gradually resorbed. The o.i. increases in thickness to 6–12 cell layers, the zones with the most intensive mitotic activity apparently being localised mainly below the two epidermal layers. The cells of its outer epidermis are rather narrow when viewed in transverse sections and show the beginning of a radial elongation. The outer cell wall is already somewhat thickened, and presumably the production of the mucilaginous layer has commenced. The o.i. contains amyloplasts. The endocarp is partly of subdermal derivation, as in *Citrus*.

*The mature seed-coat*

At maturity the seed-coat consists of both the o.i. and remains of the i.i. and the outer layers of the nucellus (*fig. 1H*). The outer epidermal layer of the o.i. forms the most characteristic seed-coat layer and consists of radially stretched, and tangentially also much elongated cells forming the fibrous exotesta sensu Corner. The peripheral cell wall layers of the epidermis form a thick mucilage reacting strongly with ruthenium red and capable of swelling appreciably in water. Below these mucilaginous layers lignified wall layers are found. After removal of the mucilage the SEM images of the surface show that the radially elongated cells possess more or less equidistant protrusions (*fig. 3A*). In longitudinal sections of these cells the outer wall appears to be unequally thickened. Between the epidermal cells there are unbranched pits which have been somewhat stretched. Between the epidermal cells and the subdermal layer there is a layer also staining strongly with ruthenium red and presumably comparable with the outer mucilaginous layers. The remainder of the o.i. consists of thin-walled parenchymatic and often compressed cells with amyloplasts; the innermost cells occasionally contain crystals. The outer two cell layers of the i.i. have been resorbed, the innermost one consists of tanniferous cells with slightly thickened walls; at the chalazal side this tanniferous layer is continuous with a likewise tanniferous tissue consisting of cells with thick and somewhat suberised walls and forming an extra, water-repellent cover. Of the nucellus only the epidermal cells provided with a conspicuous cuticle and the compressed subdermal cells remain. The nucellar remains are thicker at the chalazal side. A thin layer of cells rich in fatty substances and the compressed remains of other layers represent the vestiges of the endosperm. This endosperm layer can, as in *Citrus*, be peeled off from the inside of the testa as a thin membrane. The mature seeds resemble those of *Citrus* very much. They are about 4 mm × 6 mm × 10 mm, and pale yellow but the chalazal region is somewhat darker owing to the presence of tanniferous cells (*fig. 1J*). After desiccation the seed has a dull shine owing to the dried mucilage, when fresh and wet it is slimy and slippery to the touch. The raphe is visible as a thin ridge. The position of the cotyledons varies when viewed in cross section (see *fig. 1L*); they contain lysigenous glands. Polyembryony occurs (*Fig. 1K*)). The embryo is rich in starch but poor in fatty substances. The vascular strand in the raphe is amphicribal and branches below the tanniferous chalazal tissue. The integuments are not vascularised.

### 3.2. Citrus aurantium

The development and structure of the ovule and the seed resemble those of *Poncirus* very much. The ovule primordium is trizonate, the i.i. is of dermal, and the o.i. of subdermal derivation. A clear difference with *Poncirus* is the presence in the mature seed-coat of protrusions of the epidermis entering the mucilaginous cover (see *fig. 2a*: t.s. of the seed coat). When viewed under the light microscope these protrusions have irregular and somewhat helical cell wall thickenings. *SEM photomicrographs 3 B, C, D* show the protrusions, after the mucilaginous coat had been removed, of a seed of the edible orange (*Citrus sinensis*) on which they are beautifully developed; *fig. 3 D* also shows the epidermis cells, longitudinally stretched in the direction of the long axis of the seed between the protrusions. Below the epidermal cells with stratified and lignified walls there is, as in *Poncirus*, a substance reacting strongly with ruthenium red (*fig. 2a*). The o.i. is locally compressed and in the innermost layer there are scattered, crystal-containing cells. Of the i.i. the innermost layer is preserved; it consists of strongly tanniferous cells with somewhat thickened walls. *Fig. 2a* shows a tr.s. at about the level of the chalaza where the endosperm and nucellus layers are rather massive.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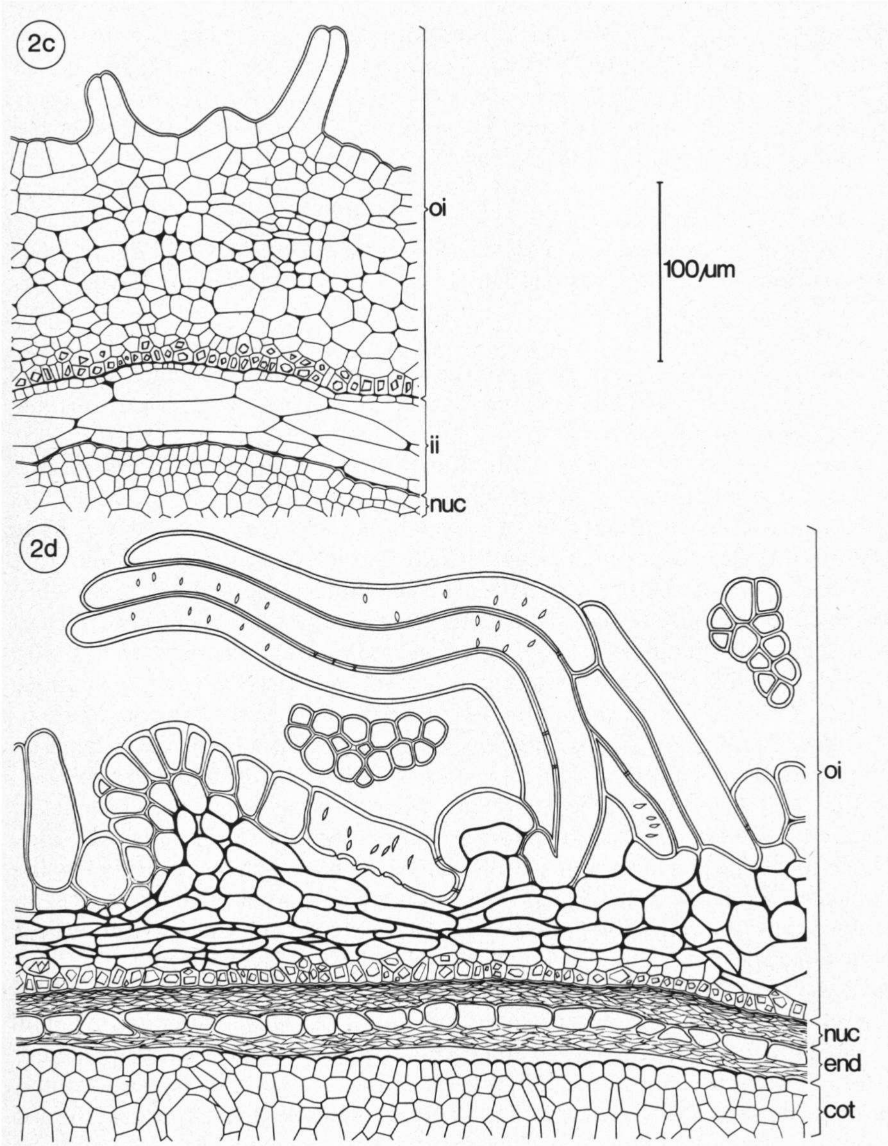



Fig. 2. a. Fully developed seed-coat of *Citrus aurantium* as seen in tr.s. of the seed;
b: same of *Citropsis* cf. *C. tanakae*;
c and d: developing and fully developed seed-coat of *Murraya paniculata* in tr.s.

The cotyledons, rich in fatty substances but poor in starch, contain small lysigenous oil glands. The seeds of *C. aurantium* are rather variable in size. In *Citrus* the testa has small ridges and folds not found in *Poncirus*, so that the seeds of the latter appear to be smoother. The ridges on *Citrus* seeds are formed by the unequal radial elongation of the dermal cells.

### 3.3. Fortunella margarita

The seeds of the kumquat resemble those of *Citrus* very closely. The dermal cells form strongly curved protrusions penetrating into the mucilaginous layer.

### 3.4. Citropsis cf. C. tanakae

The ovaries of the *Citropsis* species studied have one ovule per locule. The small, berry-like fruits contain one seed or only a few. A thin layer of clearly discernible endocarp is present.

The ovule closely resembles those of the other genera of the Aurantioideae hitherto studied. It is crassinucellate and the nucellus has a cap. When the ovule is full-grown the i.i. is about five cell layers thick and the o.i. 5–7 layers. The obturator trichomes are sometimes 2-celled. Starch is present in the nucellus top, in the i.i., and in the o.i. The cells of the outer epidermis of the o.i. begin to stretch in the radial direction. The seed-coat differs clearly from the testae of *Poncirus* and *Citrus* in some minor points: the outer cell wall layers, reacting strongly with ruthenium red and covered with a cuticle poor in cutin (Corner's "gelatinous pellicle"), form a somewhat harder and smoother surface than the comparable mucilaginous layer of *Citrus* and *Poncirus*, and the thick-walled and longitudinally stretched epidermal cells have more clearly stratified and much thicker, lignified cell walls than the comparable cells of *Citrus* and *Poncirus*. The pits in these walls are often repeatedly bifurcate especially at the upper and lower sides of the cells. The pits in the outer walls widen into funnel-shaped ends below the "pellicle"; after the pellicle has been removed these wide openings appear in surface view as a reticulate pattern (*figs. 4A, B*). In slightly oblique sections cut almost parallel to the outer surface of the testa these ends appear under the light microscope as "cytoplasm containing perforations" (*fig. 4 D*). The lower tangential cell wall contains pits not widening at their tips (*SEM photograph 4C*). Below the epidermal cells, as in *Poncirus* and *Citrus*, there are vestiges of a layer reacting strongly with ruthenium red. Below these remains, a layer of thin-walled and partly compressed cells is present. The inner side of the o.i. derivatives consists of a layer of crystal-containing cells which may become very thick; each cell contains one large crystal and a number of small ones. Chiefly below and at both sides of the raphe strand the o.i. contains cells with thick, multi-layered, and pitted walls. The i.i. is much compressed but still recognisable as a thin layer with cell wall remains devoid of tannin. Of the nucellus only a layer of compressed cells remains which is covered with a fairly thick cuticle reacting strongly with Sudan IV; this cell layer becomes thicker towards the chalazal side. Within the nucellus a usually single layer of endosperm cells is present. The chalazal tissue is tanniniferous and has somewhat thickened cell walls.

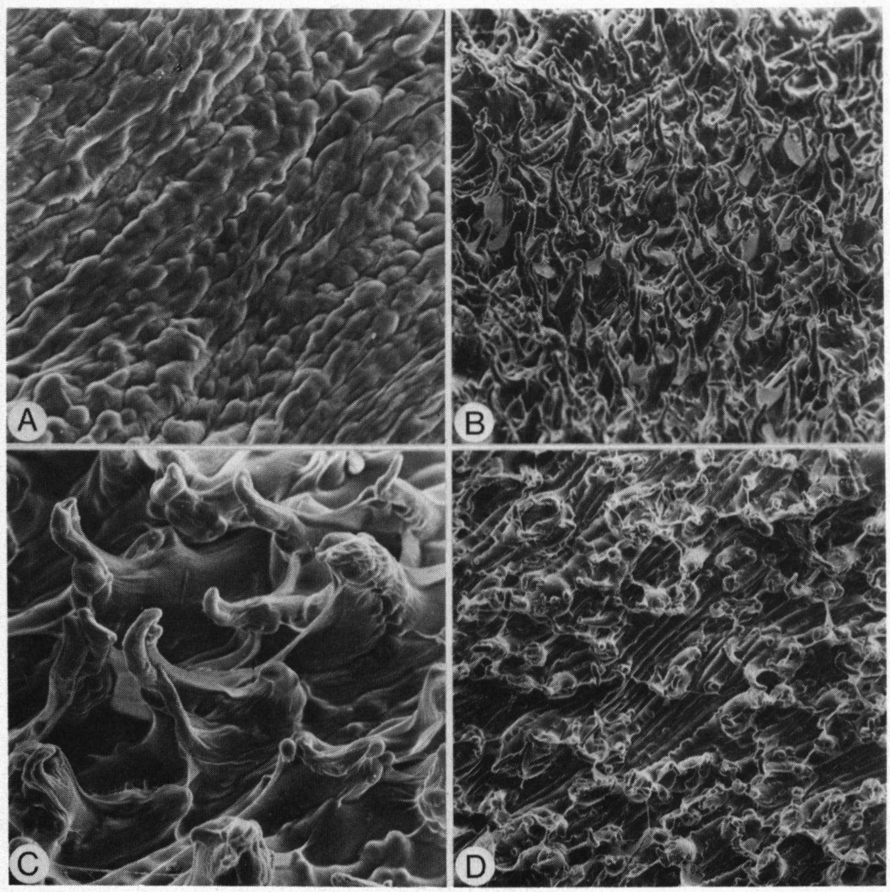
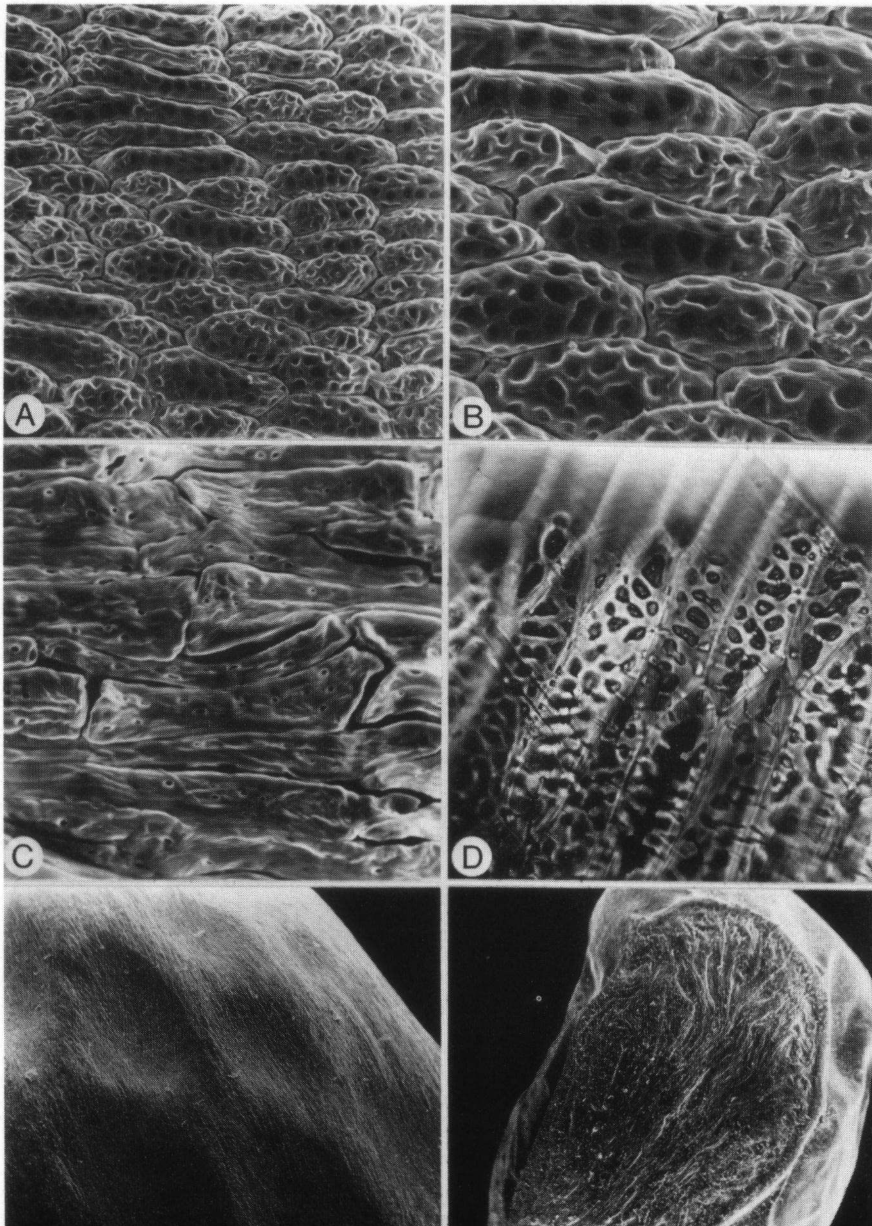



Fig. 3. S.E.M. photomicrographs of seed-coat surface, A: *Poncirus trifoliata* (c. × 600); B, C, D: *Citrus sinensis* (c. × 115, × 380. and × 120, respectively).

The mature seeds are yellowish and measure about 5 mm × 7 mm × 9 mm (*fig. 6G, H*). The surface is hard and shiny. If more seeds have developed in the same fruit they are mutually compressed. The seed has small depressions particularly at the chalazal end. The uneven surface is perhaps comparable with the ridges on *Citrus* seeds (compare *figs. 4E, 6G, H*). Above the raphal strand a groove is visible which develops by the lack of differentiation of the epidermal cells overlying the raphe bundle (*fig. 6H*). The latter is amphicribral and contains two or three separate xylem strands. The vascular bundle ramifies within the chalaza. Fig. 4F shows the chalaza after a treatment with $HNO_3$ to remove the overlying tissue. The embryo is rich in fatty substances and poor in starch. Subdermal tissues contribute towards the formation of the endocarp, which is only developed on the tangential walls of the fruit locules.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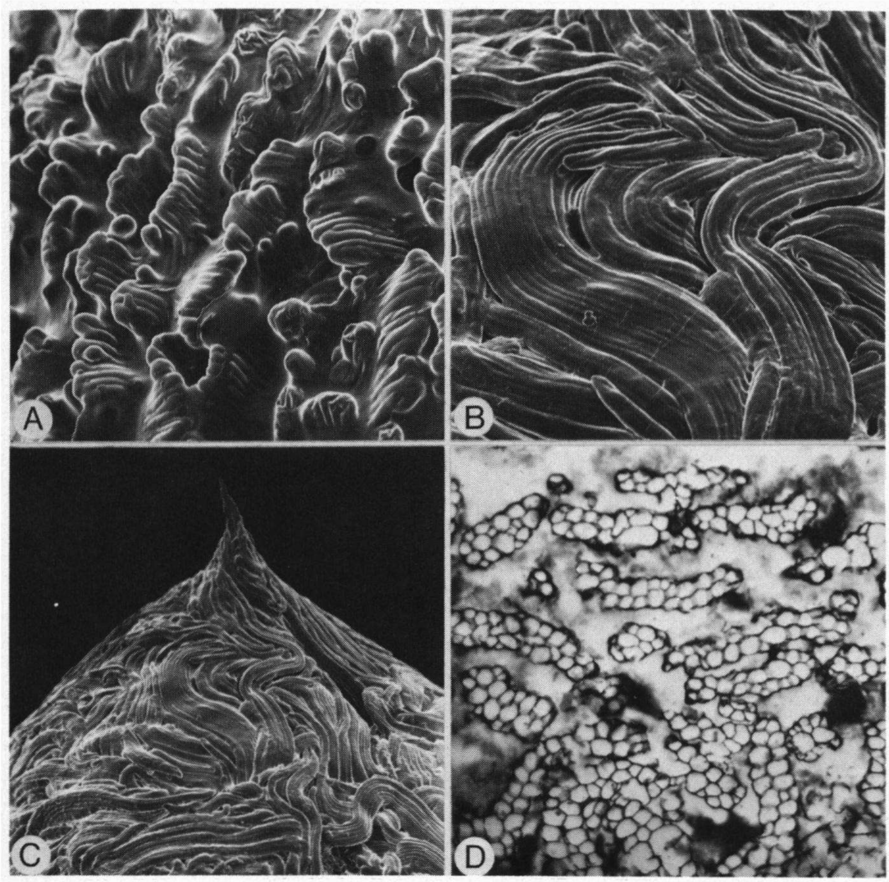


Fig. 5. S.E.M. photographs and regular photomicrograph (D) of parts of seed of *Murraya paniculata*.
A: Developing compound hairs on seed-coat (120 ×);
B: Detail of surface of fully developed seed (80 ×);
C: Micropylar end of seed (30 ×);
D: tr.s. of compound hairs (transmitted light) (150 ×).

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Fig. 4. S.E.M. photographs and regular photomicrograph (D) of parts of seeds of *Citropsis* cf. *C. tanakae*.
A and B: seed coat surface (resp. 300 × and 600 ×);
C: underside of lignified epidermal cells (600 ×);
D: section nearly parallel to the seed-coat surface (450 ×);
E: seed-coat surface (20 ×);
F: chalaza with ramifying vascular strand (after removal of outer tissue).

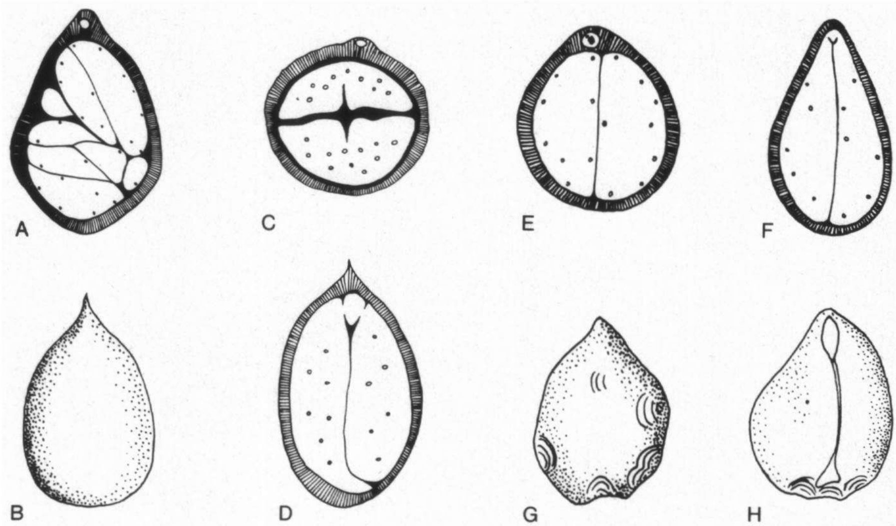


Fig. 6. A: tr.s. of seed of *Citrus sinensis* showing polyembryony;
B: seed of *Murraya paniculata;*
C and D: tr.s. and l.s. of ripe seed of *Murraya paniculata*;
E and F: tr.s. and l.s. of seed of *Citropsis* cf. *C. tanakae*;
G and H: dorsal and ventral view of ripe seed of *Citropsis* cf. *C. tanakae.*

### 3.5 Murraya paniculata

The ovary is bilocular and each locule contains two ovules. It matures into a berry-like fruit with one or two seeds. With the naked eye no endocarp emergences can be observed. The ovules are not very much different from those of the three other species studied; when it is fully developed, its i.i. is about four, and the o.i. about six cell layers thick.

During seed maturation characteristic trichomatous elements are formed by the outgrowth of groups of dermal cells of the o.i. It appears that a large number of dermal cells takes part in the formation of these protrusions. In a very young stage of development the trichomes originate as comb-like structures clearly running in the longitudinal direction of the seed (*fig. 2C, 5A*). The trichomatous elements are, at this early stage, almost perpendicular to the surface, but during the subsequent development of the seed they are squeezed against the seed-coat (*fig. 5B, C*). The trichomes in question are two cell layers thick and are built up of numerous cells as shown in the transverse sections in *fig. 5D*. In a tr.s. of a mature seed-coat the following sequence is encountered from the outside towards the centre: the above-mentioned trichomes which are covered by a cuticle poor in cutin and have slightly thickened, stratified, and unlignified cell walls. There is no mucilaginous layer. The innermost layers of the o.i. are partly compressed; the cells of its inner epidermis and some of the subdermal cells contain a single crystal each. In the mature testa there are usually no vestiges of the i.i. Below the rather thick nucellar cuticle reacting strongly with Sudan IV there is a layer of nucellar cells squeezed flat. A single layer of thick-walled endosperm cells faces the cotyledons. When mature the seed appears to be somewhat villous (*fig. 6 B*) and measures about 5 × 7 × 7 mm; its micropylar end is acuminate by the formation of a pointed tip by the trichomes (*fig. 5C*). The chalazal tissue contains tanniniferous cells with somewhat thickened and suberised cell walls. The embryo is rich in fatty substances and poor in starch; its cotyledons contain large lysigenous glands. Groups of endocarp cells, of dermal derivation according to HARTL (1958), can only be discerned microscopically; they are presumably responsible for the formation of the mucilage surrounding the developing seeds. In contrast to *Citrus*, in *Murraya* other types of endocarp vesicles do not exist (HARTL 1958).

## 4. DISCUSSION

*Poncirus*, *Citrus*, *Fortunella*, and *Citropsis* are included in the tribe Citrinae, subtribe Citreae by SWINGLE & REECE (1967). Their seed-coat structure shows a great similarity, although the testa of *Citropsis* is more complex than that of *Poncirus* and *Citrus* owing to its strongly lignified epidermal cell walls provided with branched pit canals, for which Swingle & Reece gave the qualification "hard-shelled seeds". In addition, in *Citropsis* the layer of crystal-containing cells may locally become very thick, thick-walled cells occur in the o.i., and the raphe contains a multiple xylem strand. Swingle & Reece consider *Citropsis* to be the likely ancestral type of the genus *Citrus*, preserved by isolation on the African continent. Thick-walled cells also occur in the o.i. of *Feronia* = *Limonia* (GALLET 1913, BANERJEE & PAL 1958, and CORNER 1976) and of *Triphasia aurantiola*, said to have "mesotestal fibres" according to CORNER (1976) According to Engler (ENGLER & PRANTL 1931: 355) the names *Feronia elephantum* Correa, *Feronia limonia* (L.). Swingle and *Limonia acidissima* (L.) are synonyms. *Murraya* was referred by Swingle & Reece to the taxon supposed to be the most primitive in its flower and fruit structure, *viz.*, the tribe Clauseneae (subtribe Clauseninae). *Murraya* differs from the three above-mentioned genera in its trichome-covered seeds and in the lack of a mucilaginous layer, lignified cells and a discernible i.i. in the seed-coat. Such features, in conjunction with the reduction of the number of ovules and seeds per fruit, point to a derived rather than primitive status of this genus, however. Multicellular hairs occur also in *Citrus trifoliata* (NETOLITZKY 1926), *Feronia limonia* = *Limonia acidissima* (BANERJEE & PAL 1958; CORNER 1976), *Aegle marmelos* (GALLET 1913, JOHRI & AHUJA 1957), and *Swinglea* (ENGLER in ENGLER & PRANTL 1931). In *Aegle* and *Feronia* the hairs are more perpendicular to the epidermis; in *Feronia* they even penetrate into the fruit "pulp" (CORNER 1976). Pectinate structures as found in *Murraya*, have not previously been recorded, however.

*Glycosmis* was also placed in the tribe Clauseneae, subtribe Clauseninae, by Swingle & Reece. The question whether *Glycosmis* and *Murraya*, with such a different seed morphology and structure, belong to the same taxon must certainly be put up for discussion (see also BOESEWINKEL & BOUMAN, 1978). Additional arguments for the segregation of *Glycosmis* will have to be searched for.

According to WATERMAN (1975) the Aurantioideae are derived from a phytochemical point of view because of the absence of some substances characteristic of the bulk of the Rutaceae. On the basis of phytogeographical and anatomical arguments ENGLER (1931) concluded that the Aurantioideae-Citrinae (comprising, *inter alia*, *Feronia*, *Aegle*, *Citrus*, and *Poncirus*) have descended from the more ancient rutoid forms whose carpels were still polyspermic. The primitive ovular structure of *Citrus* and *Poncirus*, with thick integuments and a subdermal o.i., is not at variance with Engler's views.

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