Morphological and anatomical studies of the seeds and seedlings of Eucalyptus citriodora and E. maculata

by

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Abstract: Morphological and anatomical aspects of seeds and seedlings of *Eucalyptus citriodora* Hook and *E. maculata* Hook were studied in detail and found to be extremely similar. However, the external characteristics of the seed, the seed coat anatomy and some features of the seedlings appeared to be very useful in the identification of these two economically important species.

In the last years, because of the increasing importance of the genus *Eucalyptus* in worldwide reforestation programs, interest has been stimulated in certain aspects of its systematics, genetics and morphology. Despite the large volume of literature devoted to the genus, there are only a few relevant studies related to the seed anatomy.

In most of the *Eucalyptus* species, the seeds are so very small that identification to the specific level is difficult, if based only on morphological features. In two species of high comercial value in Brazil, the seeds cannot be macroscopically differentiated. These are *Eucalyptus citriodora* Hook and *E. maculata* Hook and due to their importance it appeared convenient to carry out a detailed study of the morphology and anatomy of the seeds, and of the initial stages of germination.

The main purpose of this work was to find morphological and anatomical features in the seeds and seedlings which would permit us to differentiate these two species one from another, as well as to identify themwith certainty among the seeds and seedlings of other species of the genus.

MATERIAL AND METHODS

The seeds of *E. citriodora* and *E. maculata* were collected from selected trees

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at the "Navarro de Andrade" Forest Station in Rio Claro, State of São Paulo, Brazil. The observations of the surface features of fertile and sterile seeds or chaff (embryoless ovules) and of the seedlings were made under a stereo microscope with artificial light.

Determination of the mean weight of one hundred fertile seeds was done by random sampling and an analytical balance, as well as the determination of the mean percentage of fertile seeds (by weight) and the average number of fertile seeds per gram of mixed seeds. A calibrated eyepiece with a micrometrical slide was used to determine the length and width of the seeds.

The anatomy of the seeds was studied by means of microscopical observations of cross, longitudinal and paradermal hand-made sections of soaked seeds. The sections were mounted in glycerine (10%) as semi-permanent preparations (Sass, 1951).

A 1% ferric chloride solution with 0,1 N hydrochloric acid was used to determine the occurrence of phlobaphenes (Jensen, 1962) which results from the oxidation of tannins. These substances which confer a dark brown or black coloration to the seed coat, were extracted by treating the sections with sodium hypochlorite (Na Clo) before using other reagents and dyes.

Tests with chloriodide of zinc were made for the identification of cellulose and lignin (Jensen, 1962). Lignified walls were also identified by the phloroglucin test (Sass, 1951). The cuticle, the cutinized walls and the lipidic reserves became evident with Sudan III and IV (Johansen, 1940). Phloem in vascular bundles was localized with anilin blue (Jensen, 1962). The presence of calcium oxalate crystals in the seed integuments was detected by hydrochloric and sulfuric acid (Johansen, 1940). Proteinic reserves were identified by the Millon reagent (Jensen, 1962). The aleurone grains were stained by a 1% alcoholic eosine solution after fixation with 20% solution of mercuric chloride in absolute alcohol (Accorsi, 1941). Liquid ammonia was used for the identification of anthocyanin in the seedlings (Johansen, 1940).

The drawings and diagrams were made with a camera lucida adapted to a research binocular optical microscope.

Development of the seedling was observed under a stereo microscope and drawn in the different stages up to when the cotyledons unfold, which is the stage when in germination tests the seedlings are eliminated after counting (Larsen, 1965), and the epicotyl development commences. All the observations were carried out on fresh material.

For germination studies, the seeds were placed on moist filter paper in covered Petri dishes at room temperature (mean of 26 C) and light (Kaul & Ganguli, 1962).

RESULTS

External morphology of the fertile and the sterile seeds: In samples of *Eucalyptus* seeds there are, besides the impurities, three easily distinguishable shapes of seeds: apparently fertile seeds, provided with an embryo; "shape A" sterile seeds without embryo; and, "shape B" sterile seeds which sometimes may present a rudiment of embryo. Usually these seeds can be distinguished by their shape, dimensions and color. *E. citriodora* presented a mean of 73 ± 6% (by weight)

of fertile seeds which represented an average of 123 ± 7 fertile seeds per gram. The mean weight of 100 fertile seeds was 595.8 ± 7.0 mg. In *E. maculata* the values were: a mean of 61 ± 5% (by weight) of fertile seeds which represented an average of 110 ± 11 fertile seeds per gram. The mean weight of 100 fertile seeds was 673.9 ± 7.0 mg. The shape of seeds and chaff within a sample is diverse, but certain forms predominate. The basic shapes are here described and illustrated.

E. citriodora: **Fertile seeds** (Figs. 1-3) are black (ripe) or dark-brown (unripe); ovate, fusiform or navicular; dorsoventrally compressed; some keeled with a narrow coarse flange on the dorsal edge (Fig. 2), usually with a thick rudimentary terminal wing (Fig. 1); entire edges; moderately lustrous surface with fine pitting; hilum ventral, grey or white, egg-shaped, sunken; micropyle placed beneath the hilum; 2.50 ± 0.47 mm long X 1.74 ± 0.27 mm broad.

Shape A sterile seeds (Fig 5): red-brown; elongated forms, many awl-shaped, acicular and falcate; gathered in clusters (Fig. 4) losing this arrangement when compressed; highly lustrous surface with a fine network of furrows; hilum basal; 1.73 ± 0.11 mm long X 0.65 ± 0.09 mm broad.

Shape B sterile seeds (Fig. 6): red-brown; flattened and somewhat irregular; surface characteristics similar to "shape A" seeds; hilum basal; 0.98 ± 0.20 mm long X 0.64 ± 0.15 mm broad.

E. maculata: **Fertile seeds:** similar to *E. citriodora;* 3.28 ± 0.57 mm long X 2.17 ± 0.40 mm broad.

Shape A sterile seeds: similar to *E. citriodora;* 1.38 ± 0.54 mm long X 1.17 ± 0.22 mm broad.

Shape B sterile seeds: similar to *E. citriodora:* 2.04 ± 0.18 mm long X 0.58 ± 0.12 mm broad.

Internal morphology and anatomy of the fertile seeds: In both species studied no differences were observed as to the internal morphology of the seed or to the anatomy. Therefore, the following description refers to both species indistinctly.

The fertile ripe seeds are basically constituted of the seed coat and the embryo. The seed coat consists of outer and inner integuments. Inside the seed there are remnants of the nucellar tissue and generally a single layer of endosperm.

The embryo (Figs. 16 and 17) consists of the 2 cotyledons folded in a complex way (Fig. 18), bent down along the cylindrical embryo axis or hypocotyl-root axis, and developing near the shoot origin (Fig. 15). The hypocotyl, which alone represents the embryo axis occupies about 3/4 of the seed length. At its two opposite poles, the apical meristems of shoot and root develop (Fig. 15).

The apical meristem of the shoot remains at the top of the axis between the two cotyledons, and two small leaf primordia constituting an incipient plumule, are already present.

The apical meristem of the root and the root cap are almost surrounded by the still incipient clinging disc or cupuliform organ (Fig. 15). This organ is a bulge of the hypocotyl cortex.

Seed Coat antomy (Figs. 7-13): The outer integument is composed of the

outer and the inner epidermis and between them there are several middle cell layers which frequently change in number according to their position in the seed. At the ventral face, at the rudimentary wing and at the ribs and corners there is always a larger number than at the dorsal face of the seed (Figs. 7,9 and 12).

The outer cuticle is well preserved and the cells of the outer epidermis are thin-walled, rectangular in cross section, somewhat perpendicularly (Fig. 7) elongated (mainly at the wing, ribs and corners) and in paradermal section they are generally hexagonal (Fig. 8). The cell walls are impregnated with tannins.

Isolated patches of sclereids may occur scattered in the epidermal layer. The epidermal cells are filled with amorphous dark reddish-brown tannic material, often glasslike and readily soluble in water or alcohol.

The inner epidermis is made up of small closely packed cells, rectangular in cross section (Fig. 7) and pentagonal or hexagonal in paradermal section (Fig. 8), each containing a heavy cellulosic thickening of its inner periclinal wall, which constricts the lumen and leaves little space for the crystals. This epidermis is interrupted in the region of the chalaza.

The inner integument on the ventral face of the seed has slightly distinct cells and on the dorsal face, it is almost entirely resorbed (Fig. 7).

In the internal epidermis of the inner integument, a rather evident cuticle is encountered forming short rib-like projections of cutine and penetrating into the anticlinal walls of the nucellus (Fig. 7 and 9). The formation of this cuticle is supressed in the chalazal region (Fig. 12).

Remnants of the nucellar tissue, with empty and obliterated cells are present in variable amounts beneath the inner integument on the dorsal face forming a thin layer. On the ventral face there is a greater amount of these remnants, mainly beneath the chalaza, where the cellular structure is more evident (Figs. 7, 9 and 12).

The endosperm is present at the inner boundary of the seed coat as a layer of thick-walled cells, rich in proteins and oil droplets (Figs. 7, 9 and 12).

The external part of the chalaza shows a single vascular bundle, embedded in a solid, thick sheath of sclereids (Fig. 12). Internally, there is a suberized and lignified tissue made up by thin-walled cells filled with a dense reddish-brown tannic material (Figs. 10, 12 and 13). This suberized tissue is derived in part from chalazal tissue and in part from nucellar tissue.

The hilum forms a scar sunken on the ventral surface of the seed and partially overlaps the chalaza. The hilum is formed by loosely packed cells with thin walls which sometimes are broken (Figs. 10, 12 and 13). The micropyle, in surface view, is a smallhollow below the hilum cup, and surrounded by elongated thin-walled epidermal cells (Figs. 10 and 11).

Close to the upper edge of the hilum a collateral vascular bundle (the phloem overlaps the xylem on the outer side) with helically thickened tracheids penetrates the seed and extends into the chalazal sheath of sclereids, spreading horizontally in a fan-like fashion, giving rise to several branchlets (Figs. 12 and 13).

The rudimentary wing is an extension of the external layers of the outer integument (Fig. 9).

Anatomy of the embryo: The embryo axis is covered by the protoderm which is composed of more or less cubic cells (Fig. 14). Below the protoderm there is a ground meristem, precursor of the cortical ground tissue, formed by round and thin-walled cells (in cross sections), containing aleurone grains and oil droplets (Fig. 14). Arising from the protoderm of the axis as well as from that of the

cotyledons, and penetrating the cortex, there are small oil glands (Figs. 14 and 19).

Next to the ground meristem there is a cylinder of narrow and very thin-walled procambial cells, 4 or 5 layers thick, throughout the hypocotyl and cotyledons as a continuous system (Figs. 14 and 15). Internally, the ground meristem is observed again, as the precursor of the pith (Figs. 14 and 15). The cotyledons (Figs. 16 to 19) are covered by thin-walled protodermal cells, rectangular in cross section and irregular in paradermal section. On the adaxial surface, each protodermal cell contains some small aleurone grains surrounding a larger one which contains a large druse crystal of calcium oxalate. In cells of the adaxial side, these druses are smaller and scattered (Fig. 19).

The ground meristem of the cotyledon on its adaxial surface consists of 2 rows of elongate perpendicular cells, and on the abaxial side it consists of 4 or 5 layers of round cells which present small intercellular spaces. As in the hypocotyl, most of the cotyledon cells (except procambial and glandular cells) are filled with aleurone grains and oil droplets.

Germination and seedling morphology (Figs. 20 to 25): Germination and morphology of the seedlings in the two species studied are similar. When seeds are placed inside the petri dishes, the process of imbibition starts and 2 or 3 days later the rupture of the seed coat occurs at the lower end, below the micropylar region (Fig. 20). The radicle emerges, partially surrounded by the cupuliform organ. Later this organ develops long absorbing hairs and remains functional for about 30 or 40 days and then it begins to dry and exfoliate, leaving a ring-like scar. The radicle grows fast and is also covered by root hairs, shorter than those found over the cupuliform organ (Figs. 21 and 22). The hypocotyl grows simultaneously with the radicle and acquires a pink color due to the presence of anthocyanins in the epidermal cells. The hypocotyl presents on its entire surface many secretory appendages, each containing an oil droplet. These appendages are deep reddish-purple, due to the presence of anthocyanins (Figs. 21-25). They will also appear later on the epicotyl, on the cotyledonal petioles and on the leaf primordia.

After 7 or 8 days the seedling is fixed to the substratum, and soon afterwards, the seed coat is released; 15 to 20 days later the cotyledons unfold. They are reniform (Fig. 25) and present a dark reddish-purple abaxial surface and a dark-green adaxial surface. The epicotyl initiates its growth in about 25 days when the leaf primordia are developing (Fig. 25).

The older seedlings of *Eucalyptus citriodora*, when crushed, released a characteristic citric scent lacking in *E. maculata*.

Comparison among the seed length averages: As additional information to the morphological comparisons, the variation of the fertile seed length was studied in greater detail. The Kruskal-Walles technic (Sokal & Rohlf, 1969) was used to verify whether the existing difference in the average length of the fertile seeds was significant or not. According to the test, *E. citriodora* seeds do not differ significantly in length from those of *E. maculata*.

DISCUSSION

Seeds are relatively stable organs, their basic internal organization varies only slightly among related species and genera; and the differences that do exist may be

regarded as phylogenetically significant. Their superficial characteristics, such as shape, size, color and structure of the surface, often vary markedly among species and genera in the same family (Martin, 1946) so that they can be used for specific identification.
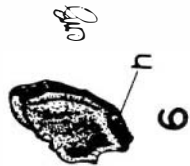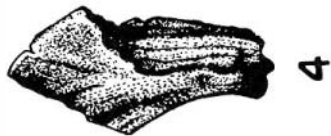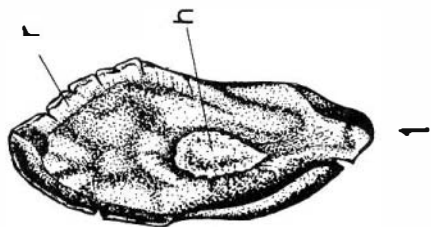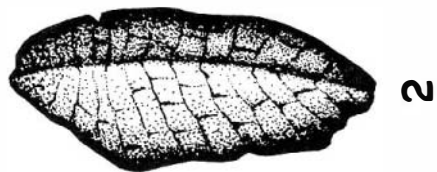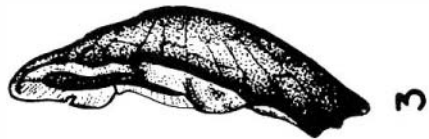
According to Gauba & Pryor (1958, 1959, 1961), Pryor & Johnson (1971) and Beltrati (1973, 1977a, 1977b) the external morphology of the fertile and sterile seeds and the seed coat anatomy in *Eucalyptus* (mainly the outer integument) are extremely valuable for the identification of the species.

In both species studied, morphological characteristics that might distinguish them were not observed, as the existing differences in the length averages of fertile seeds were not statistically significant; although, as Grose & Zimmer (1958) state, a slight variation among the characters in the different seed lots may occur. It is interesting to emphasize that these two species are closely related, being both included according to Blakely (1955) in the Series Corymbosae Peltate.

The present results lead to the conclusion that these 2 species cannot be differentiated through their seed and seedling morphology. The only difference is the lemon scent of *E. citriodora* which is missing in *E. maculata,* therefore there exists a biochemical difference. According to Larsen (1965) the citricscent is due to the presence of citronella, an essential oil produced by the glandular structures. Penfold & Willis (1966) state that *E. maculata* does not produce citronella. However, when these 2 species are compared with others previously studied (Beltrati, 1973), the embryos and seedlings present the following distinctive features: presence of 2 palisade-like rows of cells in the cotyledons; aleurone grains containing a large druse of calcium oxalate in the protodermal cells of the cotyledons; leaf primordia already developed in the embryo and the presence of glandular appendages in the seedlings. The description presented here may provide the basis for identification of the seeds and seedlings of the 2 species studied.

RESUMEN

En un estudio de las semillas y plántulas de *Eucalyptus citriodora* Hook y de *E. maculata* Hook se encontró que los aspectos morfológicos y anatómicos de ambas especies son muy similares. Sin embargo, se encontró que las características externas de la semilla, la anatomía de la cubierta seminal y algunos aspectos de la plántula pueden ser muy útiles en la identificación de estas dos especies de árboles de importancia económica.

Figs. 1-6.   *E. citriodora:-* Fertile and sterile seeds. External Morphology Figs. 1, 2 and 3, Fertile seed; Fig. 4, Sterile seed cluster; Fig. 5, Type A Sterile Seeds; Fig. 6, Type B Sterile Seeds.
(h = hilum; r = rudimentary wing).

1mm

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Figs. 7-13. *E. citriodora:*- Fertile Seed Coat Anatomy. Figs. 7 and 8, Respectively, cross and paradermal sections of the fertile seed coat (dorsal face); Fig. 9, Longitudinal Section through the rudimentary wing; Fig. 10, Seed Diagram showing the hilum, chalaza and micropyle positions; Fig. 11, Paradermal section at the micropylar region; Fig. 12, Longitudinal section through the hilum and chalaza region; Fig. 13, vascularization pattern.

(TE = outer integument; *ce* = outer cuticle; *ee* = outer epidermis; *cs* = sub-epidermal layers; *ei* = inner epidermis; *TI* = inner integument; *ci* = inner cuticle; *nu* = nucellus remnants; *en* = endosperm; *e* = embryo; *tc* = conducting tissue; *scl* = sclereid; *ch* = chalaza; *h* = hilum; *m* = micropyle).
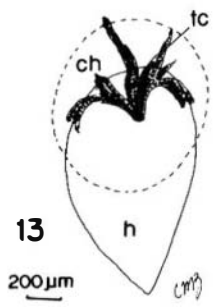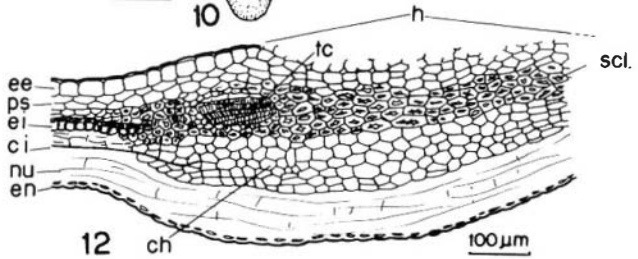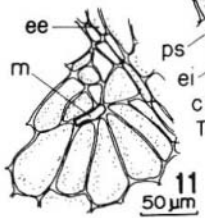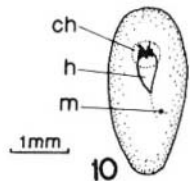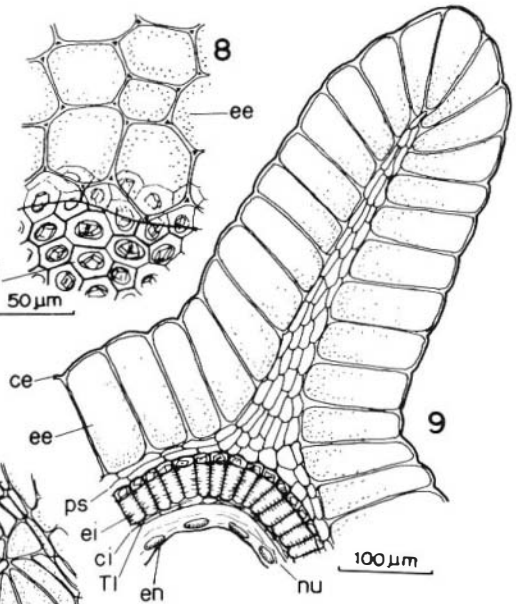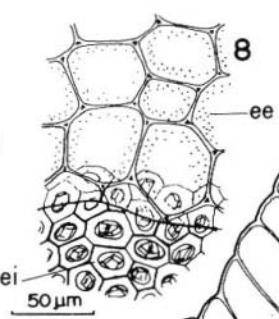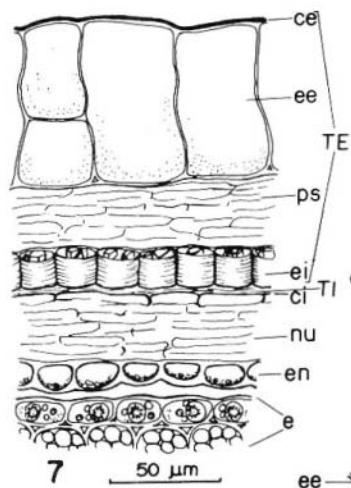
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Figs. 14-19. *E. citriodora:* Embryo. Fig. 14, Middle cross section through the embryo axis; Fig. 16, Surface view of the embryo (ventral face); Fig. 17, Surface view of the embryo (dorsal face); Fig. 18, Diagram of the embryo cross section; Fig. 19, Cotyledon cross section.
(hy = hypocotyl; pd = protoderm; mf = ground meristem; pr = procambium; gl = oil gland; cot = cotyledon; mc = apical meristem of the shoot; mr = apical meristem of the root; ct = root cap; oc = cupuliform organ; rd = radicle; tg = seed coat; ep = epicotyl).

Figs. 14–19.
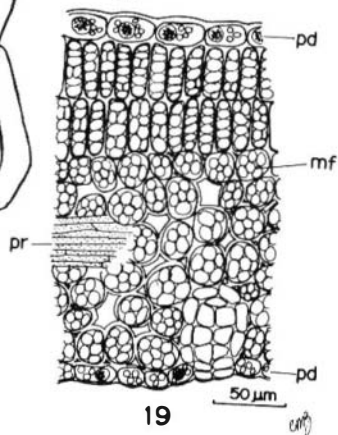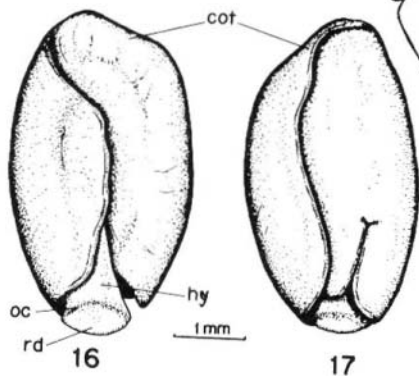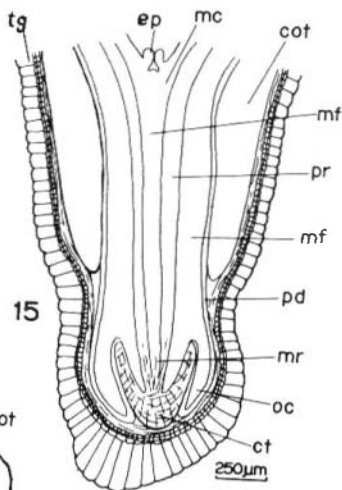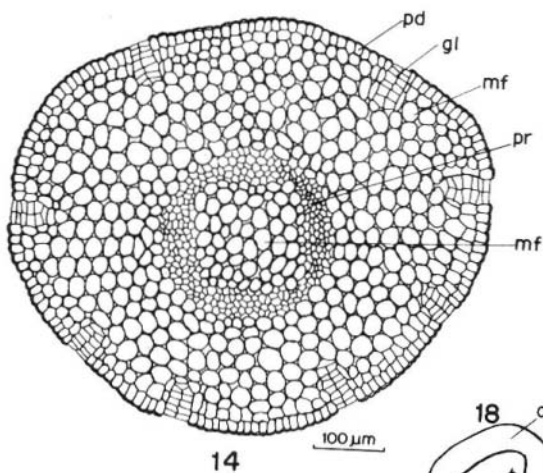
gs. 20-25. *E. citriodora:* Sequential stages of seedling development. Fig. 20, Seedling at 3 days; Fig. 21, at 4 days; Fig. 22, at 5 days; Fig. 23, at 7 days; Fig. 24, at 15 days; Fig. 25, at 25 days.

(*cot* = cotyledon; *eg* = glandular appendages; *hy* = hypocotyl; *oc* = cupuliform ●rgan; *ep* = epicotyl; *pa* = absorbing hair; *rd* = radicle; *tg* = seed coat).

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