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Structural aspects of the zygotic embryogenesis of *Acca sellowiana* (O. Berg) Burret (Myrtaceae)[1]

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RESUMO – (Aspectos estruturais da embriogênese zigótica em *Acca sellowiana* (O. Berg) Burret (Myrtaceae)). *Acca sellowiana* apresenta óvulos anátropos, bitegumentados e crassinucelados. Os tegumentos externo e interno são constituídos por duas camadas de células, exceto na região da micrópila em que têm maior número de camadas; a micrópila apresenta-se em ziguezague. O aparelho oosférico ocupa a região micropilar com sinérgides apresentando aparato fibrilar conspícuo. Na região calazal, as três antípodas estão presentes antes da ocorrência da dupla fecundação. O zigoto é formado 21 dias após a polinização controlada, e o endosperma do tipo nuclear já está presente. O zigoto sofre a primeira divisão mitótica no 24º dia. Embriões nas fases globular, cordiforme e torpedo foram observados no 30º, 45º e 60º dia após a polinização, respectivamente. O embrião maduro, caracterizado pela presença de um eixo hipocótilo-radicular bem desenvolvido e com dois cotilédones carnosos e dobrados, foi observado após 120 dias da polinização. As sementes são exospérmicas e com um único embrião do tipo espiral, característico de Myrtinae. Os estudos da embriogênese zigótica de *A. sellowiana* mostram que esta espécie apresenta características embriológicas que se adéquam ao conhecido para Myrtaceae (Myrteae, Myrtinae), e ampliam o conhecimento sobre a reprodução sexual dessa espécie nativa, cujo cultivo comercial tem sido incrementado.

Palavras-chave: embrião zigótico, feijoa, goiaba-serrana, megagametófito, óvulo

ABSTRACT – (Structural aspects of the zygotic embryogenesis of *Acca sellowiana* (O. Berg) Burret (Myrtaceae)). *Acca sellowiana* has anatropous, bitegmic and crassinucellate ovules. The outer and inner integuments are double-layered except in the micropyle, where they are composed of more layers; the micropyle is zig-zag shaped. The egg apparatus lies at the micropylar pole, and the zynergids present a conspicuous filiform apparatus. The antipodal cells are present in the chalazal region, persisting before the occurrence of double fertilization. The zygote is visible 21 days after pollination; nuclear endosperm is already present. The first mitotic division of the zygote occurs at 24th day. The globular, cordiform and torpedo embryo stages can be seen at 30, 45 and 60 days after pollination, respectively. The mature embryo characterized by the presence of a well-developed hypocotyl-radicular axis with two fleshy and folded cotyledons was observed 120 days after pollination. Endosperm is absent in the seeds, and the embryo has spiral form, characteristic of Myrtinae. The zygotic embryology studies of *A. sellowiana* indicate that this species has embryological characteristics which are in agreement with those reported for Myrtaceae (Myrteae, Myrtinae), and also broaden the knowledge about the sexual reproduction of this native species, whose commercial cultivation has been growing.

Key words: feijoa, megagametophyte, ovule, pineapple guava, zygotic embryo

Introduction

Acca sellowiana (O. Berg) Burret belongs to the tribe Myrteae (Landrum 1986), which usually is considered to be the only tribe in the subfamily Myrtoideae, and includes all Myrtaceae with inferior ovary and fleshy fruit (Schmid 1980). *Feijoa sellowiana* Berg and *Orthostemon sellowianus* O. Berg are nomenclatural synonyms (Landrum 1986), but most sources still treat this species as a member of the genus *Feijoa*. *Acca sellowiana* is a woody species, native to southern region of the Brazilian Plateau, and is also found in Uruguay and Argentina (Mattos 1990). It occurs naturally in "campos serranos" (grassland hills) in Santa Catarina State above 800 m high and is known as "goiaba-serrana". Its most common names are pinneaple guava and feijoa.

Pinneaple guava produces edible fruits (Legrand & Klein 1977). The fleshy fruit emits a spicy odor when ripe and it is used, primarily, for the production of juice, with a sweet and acidic flavor. Beyond the interest in its fruits, this species is used as an ornamental plant for its

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eye-catching flowers and leaves (Legrand & Klein 1977; Bhojwani *et al*. 1987; Canhoto & Cruz 1996a; b). As a consequence this native species is also cultivated for economic proposes.

Acca sellowiana is a species that presents a high frequency of somatic embryos *in vitro*, and it became a very important model of study for somatic embryogenesis. The induction of somatic embryos starting from mature and immature zygotic embryos has been extensively studied (Cruz *et al*. 1990; Canhoto & Cruz 1994; 1996a; b; Canhoto *et al*. 1996; Guerra *et al*. 1997; Dal Vesco & Guerra 2001; Cangahuala-Inocente *et al*. 2004; 2007). The biotechnical interest in somatic embryogenesis of *A. sellowiana* is associated with the fact that micropropagation techniques based on other somatic explant types present serious limitations (Bhojwani *et al*. 1987; Canhoto & Cruz 1996a). In addition, both sexual propagation and the conventional methods of clonal propagation, such as cutting and grafting, have also proved inadequate. The seeds germinate readily but lose their viability in a short period of time (Canhoto & Cruz 1996a), and cutting and grafting have shown low efficiency because of the effects of phenolic oxidation (Stefanello *et al*. 2005).

As noted above, somatic embryogenesis induction is limited to zygotic embryo explants, but little information is available about zygotic embryogenesis in *A. sellowiana* other than the studies by Polunina (1957; 1963) based on material grown in Russia.

Therefore, the objective of this study was to describe the zygotic embryogenesis in *A. sellowiana* grown in the region of its natural occurrence, for further comparatives studies.

Material and methods

The flower buds, flowers and fruits of *Acca sellowiana* at various developmental stages were collected at the germoplasm bank of the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (EPAGRI), Experimental Station of São Joaquim, SC, Brazil, from November 2000 to April 2001.

For embryological studies 500 floral buds were emasculated, and at anthesis these flowers were crossed by manual pollination with pollen of other individuals. Samples of 10 pistils/fruits were collected every three days during the first 30 days after pollination, and thereafter every 10 days until 120 days. Unpollinated flowers were also collected as controls.

For the histological analyses, the samples were fixed in Karnovsky's fixative (Karnovsky 1965), modified by using 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), 4% formaldehyde, and 0.2 M phosphate buffer (pH 7.2), 5:3:2 (v/v), for 4 hours at room temperature. Fixed material was dehydrated in graded-ethanol series (20-95%), and embedded in historesin (Leica®) according to the manufacturer's instructions. Longitudinal and transverse sections (5-7 μm thick) obtained with a rotary microtome, were mounted on glass slides and stained with 0,05% toluidine blue O (C.I. 52040) in 0,1 M phosphate buffer (pH 6.8) (O'Brien *et al*. 1965).

To obtain mature seeds and embryos, mature fruits (120 days after pollination) were dissected under a stereomicroscope. For scanning electron microscopy (SEM) examination, the samples were also fixed in modified Karnovsky's fixative, and dehydrated in graded-ethanol series (20-100%). These samples were subsequently submitted to critical-point drying with CO_2, and coated with gold-palladium (Silveira 1989).

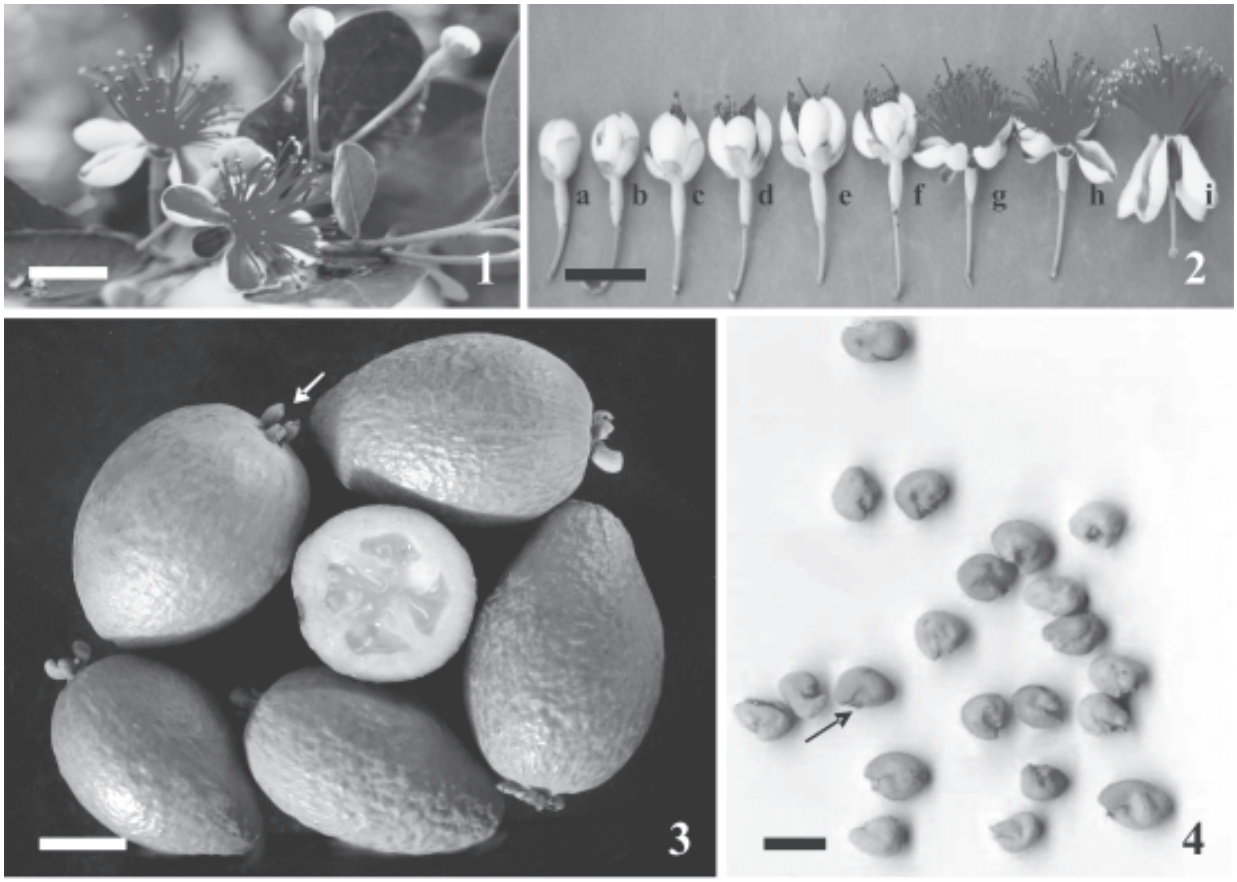
Results

Flower and fruit morphology – The inflorescence is uniflorous; flower buds and flowers at different stages occur on the same branch (Fig. 1). The successive stages of floral development in *A. sellowiana* are shown in Fig. 2 (a-h). The flowers are perfect, tetramerous, with many stamens but only one extended pistil (Fig. 2g-h). The petals are reddish on the adaxial surface and white on the abaxial, while the pistil and the stamens are reddish. After pollination the petals are reflexed and are retained for six days (Fig. 2i). About 120 days after pollination, the fruits are mature, fleshy, green, with a persistent calyx (Fig. 3), and indehiscent. Each of the four fruit locules contains numerous cartilaginous seeds (Fig. 3), which are small, reniform, and yellowish (Fig. 4).

Ovule – In flowers of *A. sellowiana*, at anthesis, the ovules are of the anatropous type (Fig. 5, and insert), with axillary placentation. They show, however, a tendency to be anacampylotropous as the connection with the funiculus is slightly laterally displaced.

The ovules are bitegmic, with the outer and inner integument composed of two cell layers except near the micropyle, where they are composed of more layers (Fig. 5-7). The micropyle is formed by the two integuments disposed in a zig-zag pattern, showing an exostome and endostome (Fig. 7). The nucellus is prominent, with many layers of cells in the micropylar region (Fig. 5-6).

In the megagametophyte, the egg apparatus occurs at the micropylar pole (Fig. 5, arrows) and is composed of two synergids (Fig. 6) and the egg cell (Fig. 8). The synergids contain dense cytoplasm, with the nucleus in the micropylar position (Fig. 6); the filiform apparatus is conspicuous (Fig. 9). The egg cell (Fig. 8) is larger than the synergids, with a conspicuous vacuole at the



Figures 1-4. Flower, fruit, and seed of *Acca sellowiana* (O. Berg) Burret. 1. Floral buds and flowers at anthesis, on the same branch. 2. Flower development: buds (a-f), anthesis (g-h), and at six days after manual pollination (i). 3. Ripe fruits (arrow indicates the calyx); in the sectioned material, four locules with seeds are shown. 4. Seeds (arrow indicates the hilum). Bars = 2 cm (1-3); 2 mm (4).

micropylar pole and the nucleus at the chalazal pole. The central cell (Fig. 8) presents a large central vacuole; the two polar nuclei are directed toward the micropylar pole. In the chalazal pole of the megagametophyte are found the antipodal cells (Fig. 10). The hypostase, zone of small dark coloured cells, is present below the antipodal cells (Fig. 10).

Post-pollination – In material collected 15 days after pollination, pollen tubes were visible next to the micropyle (Fig. 11). The penetration of the pollen tubes can be characterized as porogamic, and following the entrance of the pollen tube, the zig-zag micropyle became linear (Fig. 12, arrows).

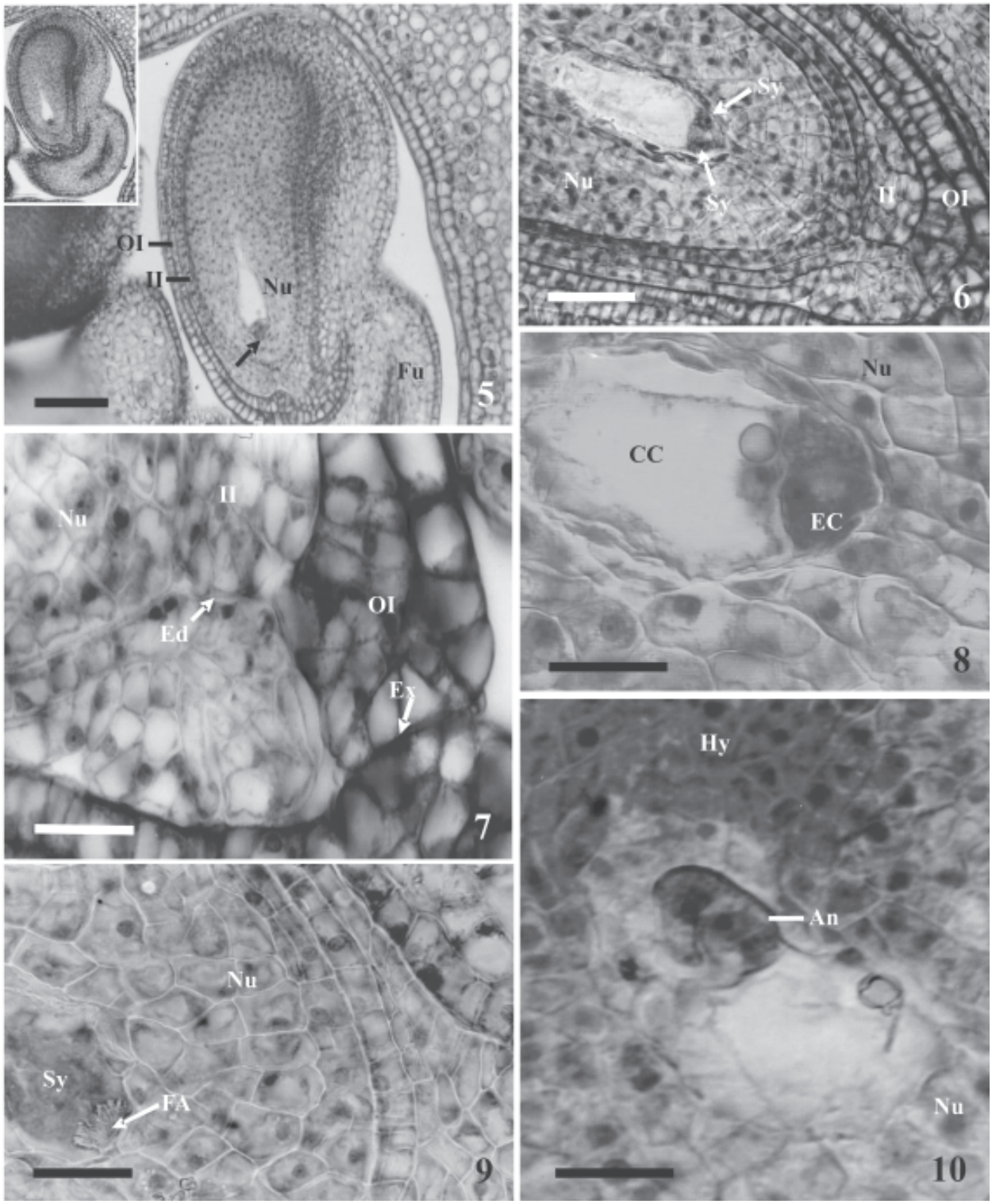
The antipodal cells degenerated before the occurrence of double fertilization. Zygote formation occurred 21 days after pollination (Fig. 13). A nuclear endosperm is already present (Fig. 13-15). At 24 days after pollination, the zygote divides and produces two cells of unequal size: the smaller cell in apical position and the larger cell in a basal position (Fig. 14).

The globular embryo (Fig. 16) is observed at 30 days after pollination with its protoderm formed. The endosperm cellularization begins at this stage, around the embryo (Fig. 16). Subsequently, about 45 days after pollination, the embryo becomes cordiform (Fig. 17-18), showing the procambial strands (Fig. 18, arrows). The suspensor is very small, and not easily seen (Fig. 18).

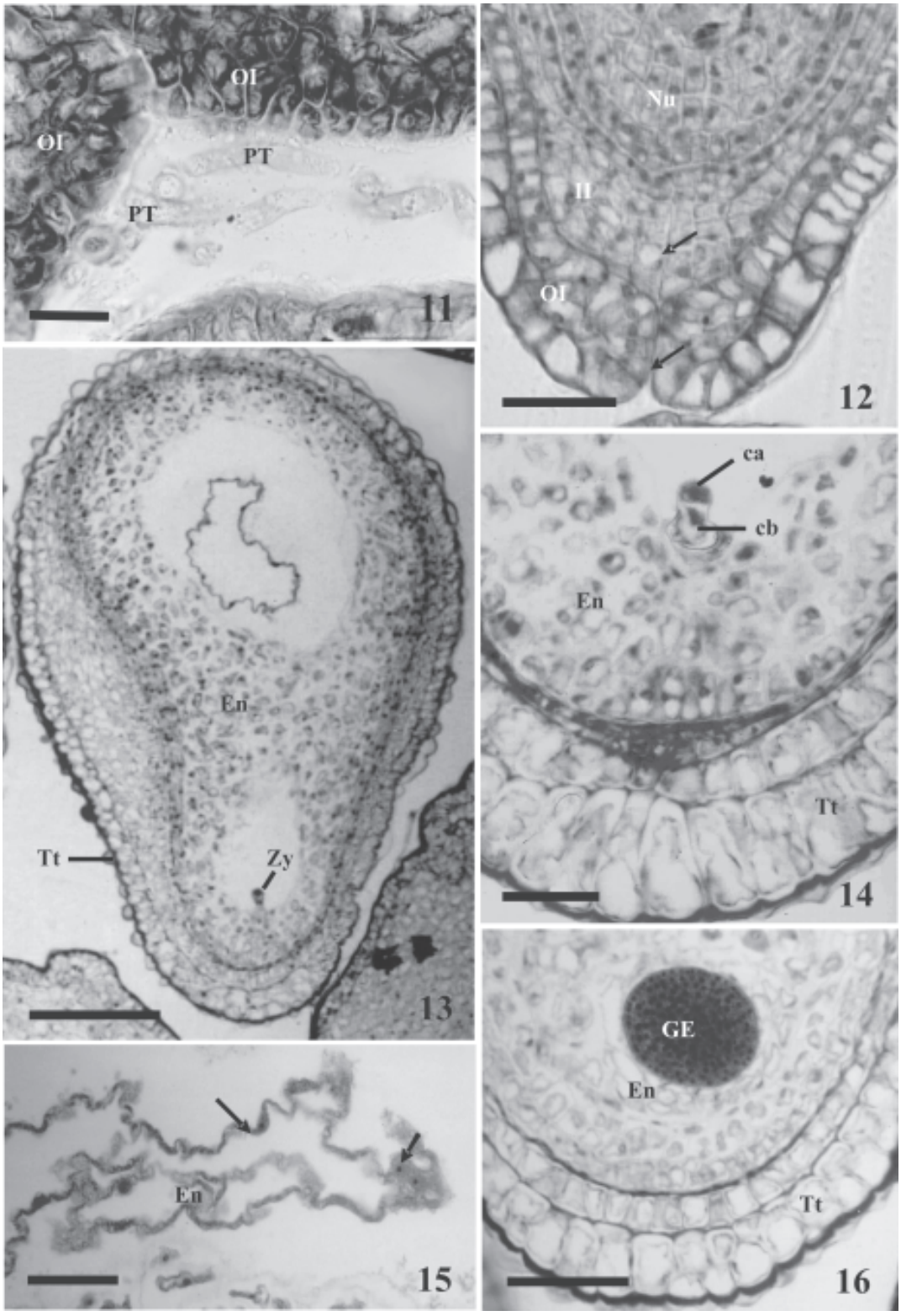
A torpedo-shaped embryo can be seen after 60 days following pollination. It is elongated, with short cotyledons (Fig. 19). The cellular endosperm is still present (Fig. 19). At 120 days after pollination, the embryo is mature (Fig. 20); this embryo is characterized by the presence of a long and relatively thick hypocotyl-radicular axis with two fleshy and folded cotyledons. The hypocotyl has a similar width to the cotyledons; the cotyledons are about as long as the hypocotyl. Each lenticular seed contains only one spiral embryo that fills the seed coat; endosperm at this stage is absent.

Discussion

In *Acca sellowiana*, the ovule is anatropous tending to anacampylotropous as a result of the ontogeny, according to the classification of Bocquet & Bersier



Figures 5-10. Ovule of *Acca sellowiana* (O. Berg) Burret, in longitudinal sections. 5. Anatropous ovule tending to anacampylotropous with the outer and inner integuments, nucellus, and the egg apparatus (arrow). In the insert, an overall view of the same ovule. 6. Detail of ovule showing the synergids at the micropylar pole, and outer and inner integuments. 7. Detail of the zig-zag micropyle with exostome and endostome; 8. Detail of the megagametophyte with the egg cell and the central cell. 9. Synergid with conspicuous filiform apparatus and well developed nucellus; 10. Detail of the megagametophyte showing the antipodal cells (two of the three cells) at the chalazal end, and the hypostase. An = Antipodal Cell; CC = Central Cell; EC = Egg Cell; Ed = Endostome; Ex = Exostome; FA = Filiform Apparatus; Fu = Funiculus; Hy = Hypostase; II = Inner integument; Nu = Nucellus; OI = Outer Integument, Sy = Synergid. Bars = 200 μm (5); 100 μm (6); 50 μm (7-10).

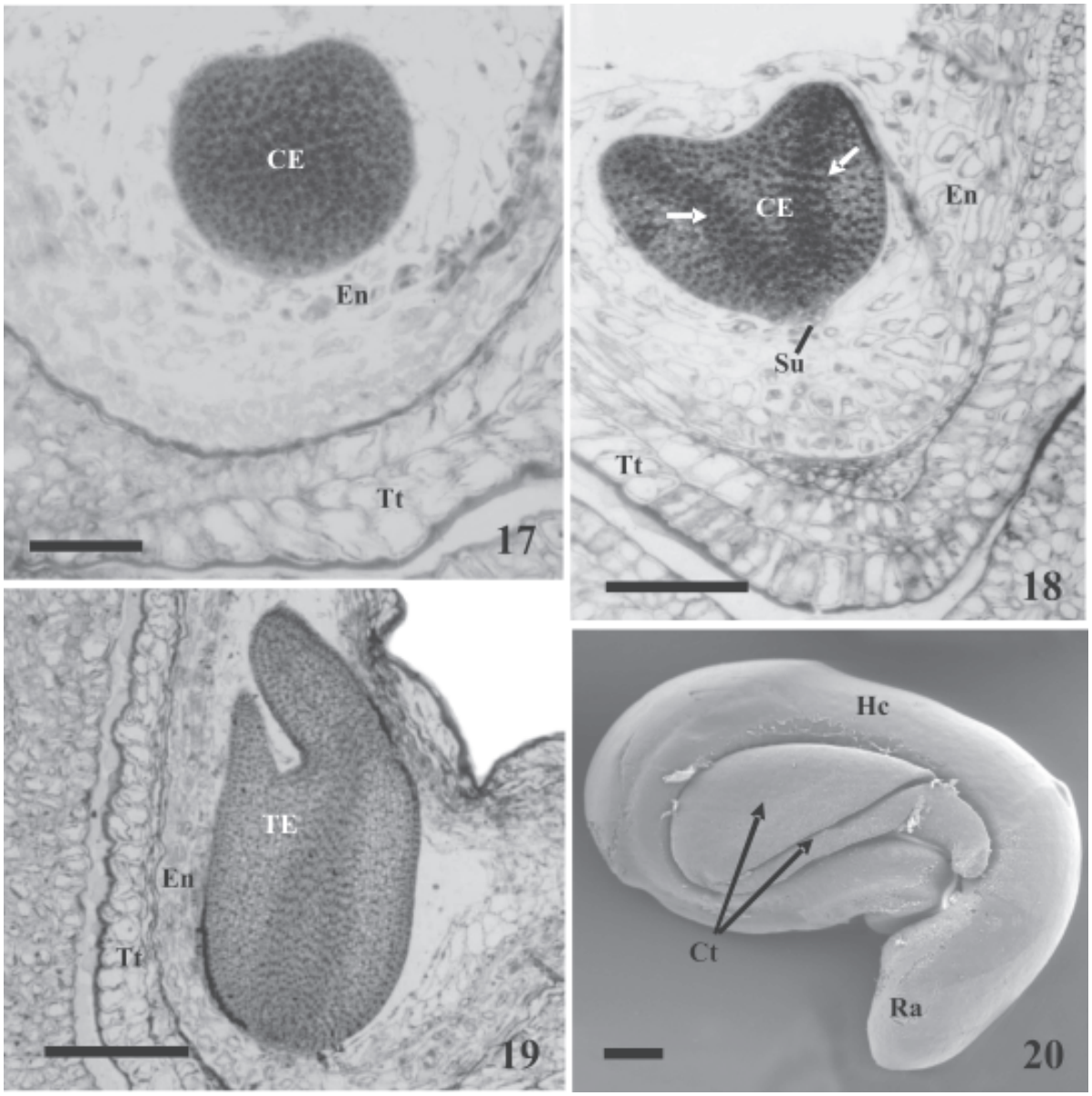


Figures 11-16. Fertilization and formation of the zygote in seeds of *Acca sellowiana* (O. Berg) Burret, in longitudinal sections. 11. Micropylar region with pollen tubes 15 days after pollination. 12. Linear micropyle (arrows) after fertilization. 13. Seed showing zygote and endosperm 21 days after pollination. 14. Micropilar region of the seed showing the basal and apical cells of proembryo, 24 days after pollination. 15. Detail of the nuclear endosperm (arrows indicate the nuclei); 16. Globular embryo surrounded by endosperm 30 days after pollination. ca = Apical Cell; cb = Basal Cell; En = Endosperm; GE = Globular Embryo; II = Inner Integument; OI = Outer Integument; PT = Pollen Tubes; Tt = Testa, Zy = zygote. Bars = 50 µm (11, 14-15); 100 µm (12, 16); 200 µm (13).

(1960). Van Wyk & Botha (1984) described hemicampylotropous ovules, sporadically tending to be anacampylotropous in *Eugenia* group, and suggested that campylotropus ovules have frequently been taken as anatropous during cursory investigation. According to Johri *et al*. (1992) ovules of the Myrtaceae are generally ana- to campylotropous.

Ovules of *A. sellowiana* are bitegmic and crassinucellate. These characteristics conform to the embryological characteristics noted by Tobe & Raven (1983) and Johri *et al*. (1992) for Myrtaceae. A multi-layered nucellus, as shown here for *A. sellowiana* was mentioned by Polunina (1957), but the figures did not illustrate this tissue.

The outer and inner integuments of the ovule of *A. sellowiana* are two layered, except in the region of the zig-zag micropyle, where each integument possesses more than two layers. Similar characteristics also occur in other Myrtaceae species: a zig-zag micropyle was observed by Prakash (1969) in *Angophora floribunda*, and the presence of additional cell layers in the integuments was mentioned for some species by Johri



Figures 17-20. Embryonic development in seeds of *Acca sellowiana* (O. Berg) Burret, in longitudinal sections (17-19) and in SEM (20). 17. Embryo at the early cordiform stage. 18. Cordiform embryo 40 days after pollination (arrows indicate the procambial strands). Note the inconspicuous suspensor. 19. Embryo in the torpedo stage 60 days after pollination. 20. Mature cotyledonary embryo 120 days after pollination; CE = Cordiform Embryo; Ct = Cotyledon; En = Endosperm; Hc = Hypocotyl; Ra = Radicle; Su = Suspensor; Tt = Testa; TE = Torpedo Embryo. Bars = 100 µm (17-18); 200 µm (19); 500 µm (20).

et al. (1992) and Lughadha & Proença (1996).

The hypostase is observed in the ovules of *A. sellowiana*, and also referred to other Myrtaceae species (Tobe & Raven 1983; Johri *et al*. 1992). Many functions have been attributed to the hypostase: this structure, besides being a storage tissue during its early development, limits extension of embryo sac in the chalazal region, establishes connection with the vascular supply and in turn stabilizes water balance and facilities nutrient transport into embryo sac during transformation of ovule into seed (Rangan & Rangaswamy 1999). However, much detailed research is needed to elucidate its precise characters in *A. sellowiana*.

Polunina (1957; 1963) described the development of the megagametophyte of *A. sellowiana* as *Polygonum* type, monosporic origin. The vast majority of Myrtaceae are *Polygonum* type (Tobe & Raven 1983; 1987), and *A. sellowiana* follows the basic pattern of this group.

In this study we noted that *A. sellowiana* synergids present a conspicuous filiform apparatus. Polunina (1957) observed the formation of the egg apparatus in the megagametophyte of this species, but this author did not mention the presence of the evident filiform apparatus in the synergids. Soverna *et al.* (2003) also observed a conspicuous filiform apparatus in the synergids of *Luma apiculata*, so, this feature may be frequent in representatives of Myrtaceae, but it has not been commented on in previous works.

According to Raghavan (1997), the filiform apparatus is structurally derived from the elaborate proliferation of cell wall material and the plasma membrane, and it may facilitate the absorption and transport of nutrients into the synergid cytoplasm. In addition, Higashiyama (2002) indicated that the amplification of the synergid wall and plasma membrane probably facilitates the secretion of chemical attractants that orient the penetration of the pollen tube into the megagametophyte. In *A. sellowiana*, where several layers of nucellus cells form a barrier to entry to the megagametophyte, it is possible that the prominent filiform apparatus facilitates the transport of nutrients to the synergids and/or egg cell, or assist penetration by the pollen tube.

The antipodal cells degenerated before the occurrence of double fertilization in *A. sellowiana*. Polunina (1957) also noted that antipodal cells are ephemeral. The presence of ephemeral antipodal cells is a characteristic of the Myrtales (Tobe & Raven 1983). In many eudicots, the degeneration of the antipodal cells occurs before or during the maturation of the megagametophyte, but, in other species, these cells persist during the entire formation of the embryo and the endosperm. The great variation in the number, cytological and morphological characteristics of the antipodal cells in angiosperms do not permit their use in the determination of types of megagametophytes (Raghavan 1997).

In *A. sellowiana*, the formation of the zygote occurs at 21 days after controlled pollination, and the cellular division, at day 24, produces the apical and basal cells. According to Polunina (1957), the apical cell and derivatives plays a major role into the development of the embryo proper and the basal cell gives rise to the suspensor, described as unicellular. The embryogeny conforms to the Solanad type in this species (Polunina 1963; Johri *et al*. 1992), and the occurrence of very short suspensor is mentioned by Tobe & Raven (1983; 1987) for Myrtaceae.

Our results showed that the globular, cordiform, torpedo and cotyledonary embryos occur at 30, 45, 60 and 120 days after pollination. Therefore the development of the zygotic embryos grown in the region of its natural occurrence was faster than the observed by Polunina (1957) for plants cultivated in the field. Polunina (*l.c.*) described the embryo development with her material derived from plants cultivated in field vs. greenhouse in Russia. The development was faster in material from the fields, with the globular embryo, and mature embryo formation occurring at 50 and 144 days after pollination, respectively. According to that author, the environmental conditions such as temperature, humidity, light and day length may cause different results, and our studies support this view.

The mature embryo of *Acca sellowiana* is spiral and characterized by the presence of a long and relatively thick hypocotyl-radicular axis with two fleshy, long, slightly leafy, and folded cotyledons and consequently, its characteristics are in agreement with those described for this species by Landrum (1986), and Landrum & Kawasaki (1997). Also in *Acca* the cotyledons are about as long as the hypocotyl, and in *A. sellowiana* they are slightly leafy (Landrum 1986).

In Myrtoideae, early embryogenesis is relatively uniform but final embryo morphology varies widely across the genera (Lughadha & Proença 1996). Embryo structure has been an important criterion in the classification of the tribe Myrteae (Landrum 1986; Landrum & Stevenson 1986) or Myrtoid clade (Wilson *et al*. 2001). According to these authors, the great majority of Myrteae can be divided into three groups based on embryo morphology. In Eugeniinae, the cotyledons are thick and fleshy, and the hypocotyl is relatively insignificant. The cotyledons are leafy and much broader than the hypocotyl in Myrciinae, whilst in the Myrtinae the embryos have a well developed hypocotyl and relatively small, narrow cotyledons. The

hypocotyl may be longer than the cotyledons, or about equal them in length; also the hypocotyl may have a diameter equal to the width of the cotyledons or it may be greatly swollen. The embryos are either C-shaped or spiral, but some embryos may be ellipsoidal to subglobose.

The endosperm of *A. sellowiana* is *ab initio* of the nuclear type, and it is present at zygote formation. Polunina (1957) observed that the fusion of the male gamete with the egg-cell occurred after the presence of 4-8 nuclei of endosperm. Our observations also indicate that the initiation of endosperm occurs before zygote formation, and its cellularization begins during the development of the globular embryo. The development of the endosperm, therefore, follows the nuclear type and it becomes secondarily cellular.

In *Acca sellowiana*, the endosperm persisted in seeds until the torpedo-shaped embryos; in mature seeds (120 days after pollination) this tissue is absent. So, the endosperm is totally digested by the embryo. Landrum (1986) noted that in *A. sellowiana* the mature seed does not contain endosperm, and Canhoto *et al.* (1996), also characterized the seeds of this species as exospermic, with the reserves in the cotyledons and the hypocotyl-radicular axis of the embryo. According to Schmid (1980), Tobe & Raven (1983) and Lughadha & Proença (1996), exospermic seeds are found in Myrtaceae.

Our studies also indicated that the zygotic embryos of *A. sellowiana* develop via similar stages to those reported for *in vitro* somatic embryos by Canhoto & Cruz (1996b), Canhoto *et al.* (1996), Canhoto *et al.* (1999) and R. Pescador (unpublished). However, zygotic and somatic embryos can show structural differences. At the cotyledonary stage since the zygotic embryo becomes spiral whilst the somatic embryo is always erect. It is also important to mention that during zygotic embryogenesis the nucellus and endosperm are formed, structures which are absent in somatic embryogenesis. According to Dodeman *et al.* (1997), comparison between zygotic and somatic embryogenesis can be made from the globular stage.

This study on zygotic embryogenesis of *A. sellowiana* indicate that this species has many embryological characteristics of (Myrteae, Myrtinae) Myrtaceae, and also broaden the knowledge about the sexual reproduction of this native species, whose commercial cultivation has been developing.

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