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Embryological features and bacterial transmission to gynoecium and ovule in *Myrsine laetevirens* (Myrsinaceae)

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SUMMARY

The gynoecium of *Myrsine laetevirens* (Mez) Arechav. consists of a five-lobed stigma, a short style and a unilocular ovary with a conspicuous free central placenta. Three to four ovules are originated from the placenta. The ovules are hemianatropous, unitegmic and tenuinucellate. The archesporial cell acts as the megaspore mother cell and divides into a linear or T-shaped tetrad. The embryo sac development corresponds to the Polygonum type. At anthesis, the placenta occupies the locule almost entirely and partially surrounds the ovules. The placental epidermal cells secrete a mucilaginous protein–polysaccharide substance in which populations of Gram-negative bacteria have been detected. During anthesis, the placenta forms grooves and canals which increase the secreting area. Bacteria were also observed in the mucilage found in the micropyle.

Key-words: bacteria, embryo sac, gynoecium, *Myrsine laetevirens*, ovule, placenta.

INTRODUCTION

Approximately 250 species are recognized in the genus *Myrsine* L., one of the 32 genera of Myrsinaceae. *Myrsine* is a pantropical dioecious genus and *M. laetevirens* (Mez) Arechav. is one of the neotropical species found in forest in the southernmost region of South America.

Data on embryology and ovular morphology in the Myrsinaceae are scarce and restricted to studies of a few taxa, i.e. *Aegiceras* Gaertn, *Ardisia* Sw., *Embelia* Burm, *Myrsine* and *Maesa* Forssk (Dahlgren 1916; Davis 1966; Johri *et al.* 1992). The ovule is anatropous, tenuinucellate and bitegmic in *Ardisia*, *Embelia* and *Maesa* (Dahlgren 1916; Netolitzky 1926; Schürhoff 1926; Sankara Rao 1972; Philipson 1974; Corner 1976; Dahlgren 1980; Johri *et al.* 1992) or unitegmic in *Aegiceras* (Carey & Fraser 1932; Philipson 1974; Corner 1976), with the micropyle formed by the inner integument in the case of the bitegmic species. It is also known in the Myrsinaceae that the archesporial cell functions directly as the megaspore mother cell and the chalazal megaspore of the linear tetrad develops into a Polygonum type embryo sac enclosed by an endothelium (Davis 1966; Johri *et al.* 1992).

Some embryological aspects of *Myrsine africana* have been studied by Dahlgren (1916). Although this species is assumed to be bitegmic (Anderberg & Ståhl 1995), there has been no anatomical study concerning this topic.

The development and structure of the ovary in Myrsinaceae have not been studied and the occurrence of a secretory placenta surrounding the ovules has been described only in *Ardisia* (Miller 1990).

One of the implications of the embryological investigation carried out in this study was the finding of colonies of bacteria living in the ovary of the pistillate flowers of *M. laetevirens*. This is the second such report in the family. Colonies of bacteria in the ovary had previously been found in the genus *Ardisia* (Miller 1990).

In this paper we present the general features of the ovary, ovule and embryo sac of *M. laetevirens*. Pistillate and staminate flowers are comparatively analysed. The presence of the colonies of bacteria living inside the ovary are described and compared with those of *Ardisia crispa* Thunb. A. DC.

MATERIALS AND METHODS

Source of material

Pistillate and staminate flowers were obtained from a natural population of *M. laetevirens* growing in the marginal rain forest of Punta Lara, Argentina.
Myrsine laetevirens: Argentina, Prov. Buenos Aires, Punta Lara, Jan. 94. Otegui 51, 55, 56 (LP).

Specimen preparation

Light microscopy (LM). Whole flowers and small blocks of tissues cut from the ovaries were fixed in formalin:propionic acid:ethyl alcohol (0·5:0·5:9) for 2 days, dehydrated through an ethanol series and embedded in Paraplast. Serial sections, 5–8 mm thick, were cut and stained with periodic acid-Schiff (Pearse 1985) and counterstained with toluidine blue O (O'Brien & McCully 1981).

Callose was detected with aniline blue 0·01% (Gahan 1984). The slides were examined in a Nikon Microphot FX with the BV filter (main wave length 436 nm, exciter filter EX 400~440, dichroic mirror DM 455, barrier filter BA 470). Proteins were detected with Coomassie brilliant blue (Gahan 1984). The samples that had been embedded for TEM were also used for LM.

Transmission electron microscopy (TEM). Samples were fixed in 2·5% glutaraldehyde solution in 0·1 M phosphate buffer, pH 7·5, vacuum infiltrated for 2 h, rinsed three times in the same buffer and then postfixed for 3 h in 1% osmium tetroxide. These samples were dehydrated in an ethanol–acetone series and embedded in Spurr's resin. Sections were mounted on grids, stained with uranyl acetate followed by lead citrate and examined with an EM 109 Turbo Zeiss transmission electron microscope.

Scanning electron microscopy (SEM). Dissected placentas and ovules fixed in formalin: propionic acid:ethyl alcohol (0·5:0·5:9), were dehydrated in ethanol–acetone series, critical point dried using liquid CO_2 (Jefree & Read 1991), mounted on stubs, coated with gold-palladium and examined with a Jeol JSM-T 100.

Bacterial identification. Gram-reagent (Locquin & Langeron 1985) was applied directly on the mucilage from ovaries of pistillate flowers.

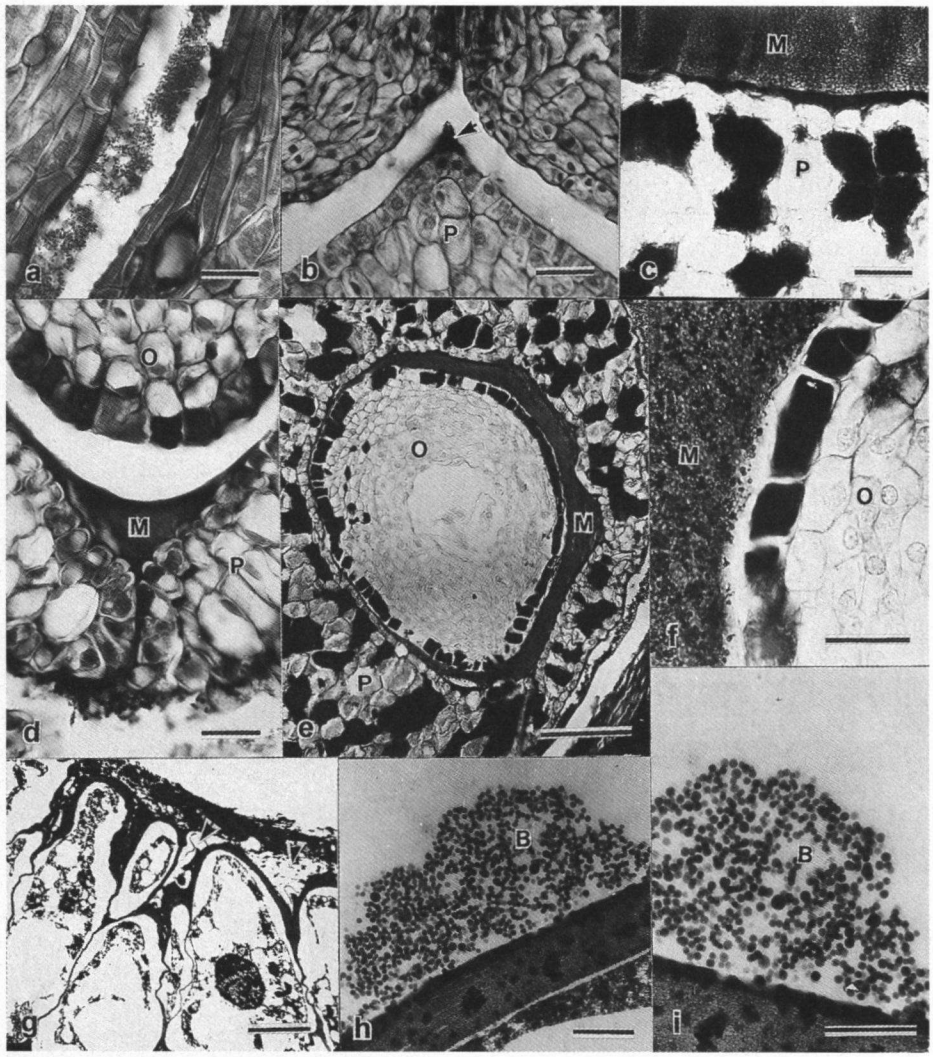

Fig. 1. Mucilage and bacteria in the gynoecium of the pistillate flowers. (a)–(g) LM photomicrographs. (a) Mucilage in the stylar canal. Scale bar: 20 μm. (b) Mucilage (arrow) in the sub-stylar chamber. Scale bar: 20 μm. (c) Placental epidermis secreting mucilage. (d) Canal at the base of the placenta. Scale bar: 20 μm. (e) Ovule immersed in the placenta. Scale bar: 100 μm. (f) Mucilage containing bacteria. Scale bar: 50 μm. (g) Section of the placenta showing the grooves (arrows). Scale bar: 10 μm. (h) and (i) TEM photomicrographs. Bacteria on the placenta. Scale bar: 5 μm. B, bacteria; M, mucilage; O, ovule; P, placenta.

RESULTS

Pistillate flowers

Gynoecium. This consists of a conical five-lobed stigma, a short style and a unilocular ovary with a conspicuous free central placenta. The placenta occupies most of the locule extending almost to the apex, where a small substylar chamber is formed; this chamber continues into the stylar canal (Fig. 1a, b).

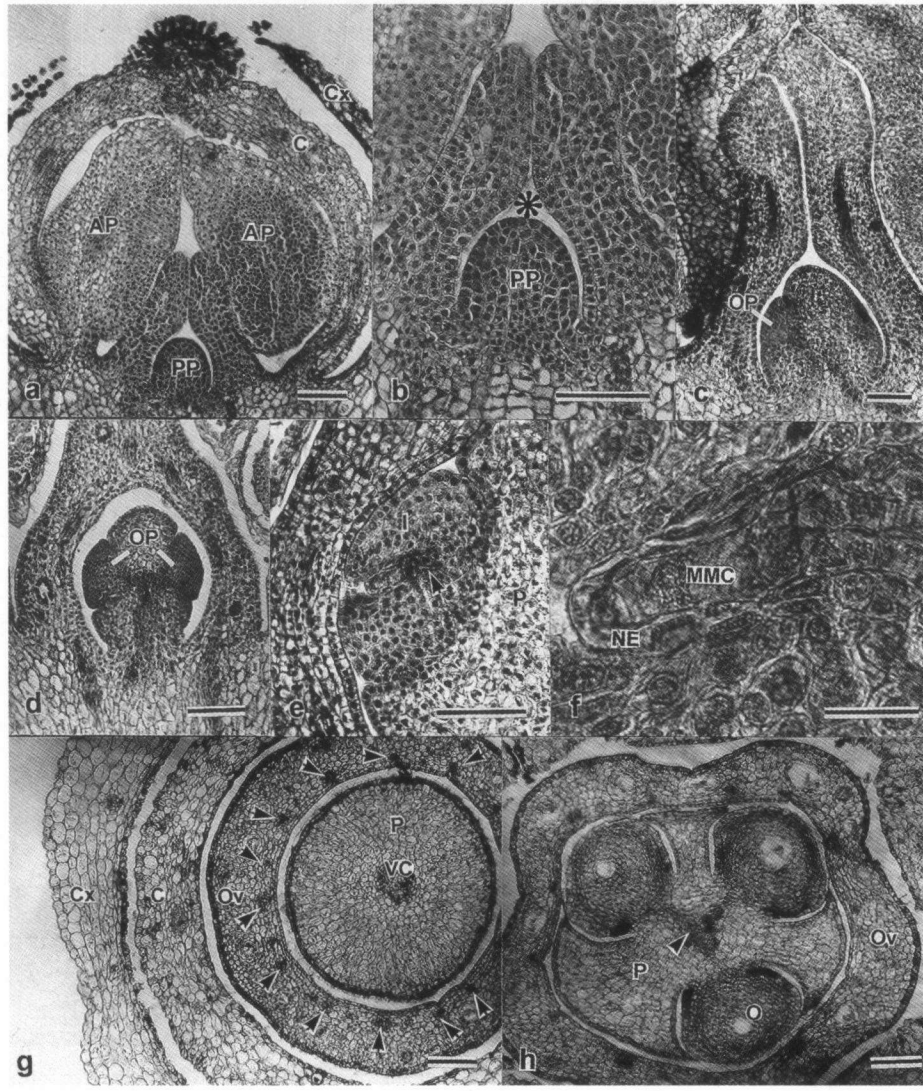

Fig. 2. Gynoecium and ovule development in *Myrsine laetevirens*. (a) Longitudinal section of a primordial pistillate flower. Scale bar: 100 μm. (b) Detail of the placenta primordium and the substylar chamber (asterisk). Scale bar: 100 μm. (c) and (d) Development of the ovule primordium from the second layer of the placenta. Scale bar: 100 μm. (e) Developing ovule; hypostase is indicated by an arrow. (f) Megaspore mother cell surrounded by a one-layered epidermis. Scale bar: 20 μm. (g) and (h) Transverse sections of pistillate flower. (g) Section made in the base of the ovary; black arrows: vascular bundles supplying the ovary wall. Scale bar: 100 μm. (h) Section made at the middle of the placenta. The vascular cylinder ramifies to supply the ovules (arrows). Scale bar: 100 μm. AP, anther primordium; C, corolla; Cx, calyx; I: integument; MMC, megaspore mother cell; NE, nucellar epidermis; O, ovules; OP, ovule primordium; Ov, ovary; P, placenta; PP, placenta primordium, VC, vascular cylinder supplying the placenta.

In the flower primordia, the gynoecium is originated as a ring surrounding the developing placenta (Fig. 2a, b). Above the placenta, the ring decreases in diameter and extends vertically, forming the style (Fig. 2c). The ring closes at the top of the style, and finally a five-lobed stigma is formed.

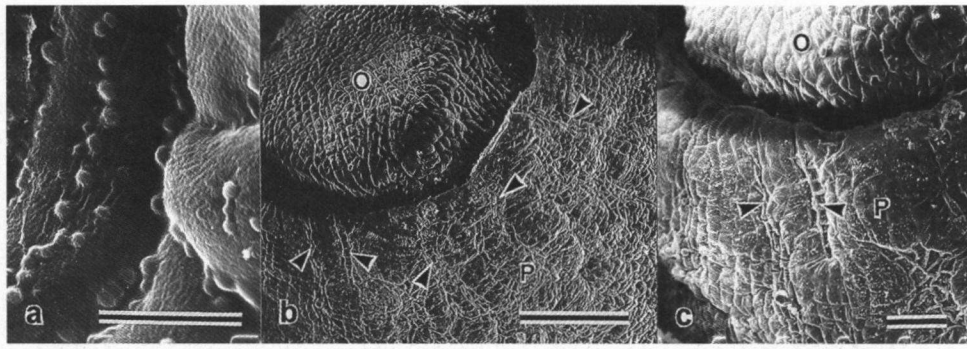

Fig. 3. SEM photomicrographs of the placenta in pistillate flowers. (a) Epidermal cells secreting the mucilage. Scale bar: 10 μm. (b) and (c) Grooves (arrows) terminating at the margins of the placenta that surround the ovules. Scale bar: 100 μm. O, ovules; P, placenta.

The ovary wall is supplied by 15 vascular bundles running in close proximity to the locule (Fig. 2g). Five symmetrically arranged vascular bundles extend into the style up to the stigma base. The vascular supply of the placenta comes up from the base of the ovary as a continuous vascular cylinder (Fig. 2g), which runs along the placenta and then branches out to supply the ovules (Fig. 2h).

During anthesis, the epidermal cells of the placenta secrete a mucilaginous substance which accumulates between the placental surface and the ovarian wall (Figs 1c, 3a). The mucilage contains polysaccharides and proteins. The presence of polysaccharides was determined by staining with PAS, whereas the presence of protein was determined by staining with Coomassie brilliant blue. Populations of Gram-negative bacteria were also detected in the mucilage secreted by the placenta (Fig. 1h, i).

The placenta develops surrounding the ovules. This placental growth is produced by the increase in size of the parenchyma cells and by anticlinal divisions of its epidermal cells, the cells of the placental epidermis then become column-shaped, and at certain points their radial adjoined walls detach from one another. This causes the grooves to form on the placental surface (Figs 1g, 3b, c). These grooves end at the margins of the placenta that surround the ovules (Fig. 3b, c). Also, canals which run through the placenta are formed facing the micropyle of the ovules (Fig. 1d). All the epidermal cells, including those of the grooves and canals, are secretory cells and produce abundant mucilage.

Ovules. Three to four ovules originate in the placenta; they are more or less sessile, without a distinct funicle (Fig. 2e), hemianatropous, unitegmic and tenuinucellate (Figs 1e, 4a, b, c). The ovule primordium (Fig. 2c, d) is initiated by periclinal divisions in the subdermal layer of the placental meristem, therefore it is a two-zonate primordium (Bouman 1984). The nucellus consists of a one-layered epidermis and one archesporial cell (Fig. 2f). The archesporial cell is conspicuous due to its large size, its denser cytoplasm and prominent nucleus (Fig. 2f). The archesporial cells acts directly as the megaspore mother cell (MMC).

At the time of anthesis, the integument is eight to twelve cells thick (Fig. 4b). It is initiated in the subdermal layer of the ovular primordium (Fig. 2e). The micropyle is narrow and long (Fig. 4b).

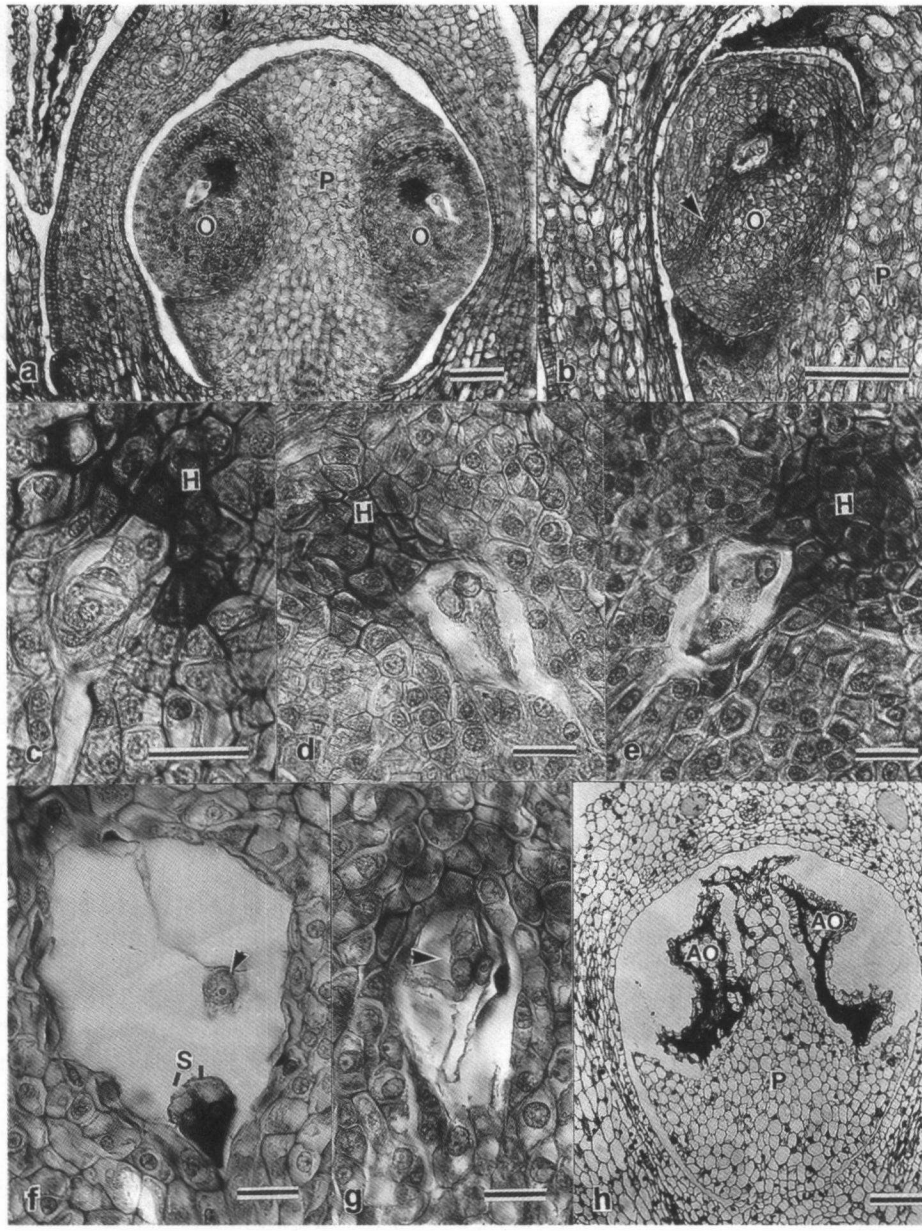

Fig. 4. Ovule, placenta and embryo sac development. (a) Placenta occupying the ovary cavity almost entirely and partly surrounding the ovules. Scale bar: 100 μm. (b) Longitudinal section of an ovule with a narrow micropyle (arrow). Scale bar: 100 μm. (c)–(g) Megasporogenesis and embryo sac development. Scale bar: 100 μm. (c) T-shaped tetrad of megaspores. Hypostase consisting of thick-walled cells with positive PAS-reaction and dense cytoplasm. Scale bar: 20 μm. (d) Young coenocytic embryo sac with two nuclei. Scale bar: 20 μm. (e) Young coenocytic embryo sac with the two nuclei at the opposite poles. Scale bar: 20 μm. (f) Synergids and the central cell with its already fused nuclei (arrow). Scale bar: 20 μm. (g) Antipodal cells (arrows). Scale bar: 20 μm. (h) Longitudinal section of the ovary of a staminate flower with aborted ovules. Scale bar: 100 μm. AO, aborted ovules; H, hypostase; O, ovules; P, placenta; S, synergids.

A hypostase is differentiated; it consists of a group of cells with dense cytoplasm and thick cell walls, which stain with the PAS-reaction (Figs 2e, 4c–e). A single bundle runs through the funicle and ends next to the hypostase.

Megasporogenesis and embryo sac development. The MMC divides meiotically and gives rise to a linear or T-shaped tetrad (Fig. 4c). During the megasporogenesis, the callose is deposited on the cell walls of the megaspores, especially on the walls separating one megaspore from another. The three micropylar megaspores degenerate and the chalazal megaspore remains functional. The functional megaspore enlarges and its nucleus divides mitotically (Fig. 4d). The nuclei migrate to each pole (the micropylar and chalazal poles) of the cell and a central vacuole is formed (Fig. 4e). Two mitotic cycles occur, subsequently forming a tetra- and an octo-nucleate coenocytic embryo sacs. The mature embryo sac is seven-celled. This development corresponds to the Polygonum type. The egg cell and the synergids form the egg apparatus. The pear-shaped synergids have a filiform apparatus at the micropylar pole. Most of the cytoplasm and nucleus of the synergids are located in the micropylar half of these cells, whereas a vacuole occupies the chalazal pole (Fig. 4f). The egg cell has a single large vacuole in the micropylar half and most of the cytoplasm occupies the chalazal pole. The two polar nuclei fuse at the centre of the central cell; before anthesis, the resulting diploid nucleus migrates close to the egg apparatus (Fig. 4f) and a very large vacuole occupies the centre of the cell. The three antipodal cells are uni-nucleate, with small vacuoles, and dense cytoplasm (Fig. 4g); almost immediately after fertilization, the antipodals degenerate.

Bacterial populations. The flower primordium is covered by the bracts of the inflorescence. The epidermis of the bracts have trichomes secreting mucilage. The mucilage is deposited between the young flower parts of calyx and corolla, reaching the gynoecium, which at this stage is still an open ring. Because of the closure of the gynoecium, the mucilage is trapped in its interior and is located in the stylar canal (Fig. 1a). Later, it flows towards the substylar chamber of the ovary (Fig. 1b). At this stage, populations of Gram-negative bacteria were detected in the mucilage.

The bacteria-containing mucilage flows out over the surface of the placenta along the grooves and canals, towards the micropyles (Fig. 1d–e). Bacteria were observed in the mucilage found in the micropyles.

Staminate flowers

The first steps in the development of the gynoecium and the ovules of the staminate flowers are similar to those of the pistillate flowers up to the formation of the archesporial cell. In staminate flowers the archesporial cell does not divide. Due to this interruption of the megasporogenesis, the growth of the ovule is cut off (Fig. 4h).

In the flower primordium, the trichomes of the bracts secrete mucilage but bacteria are always absent. In the ovary, the placenta is rudimentary and does not secrete mucilage.

DISCUSSION

Little is known about the ovary and ovule structure in Myrsinaceae. According to Anderberg & Ståhl (1995), the absence of septa in the ovary of Primulales has contributed

to the difficulty in determining the actual number of carpels. On the basis of anatomical features and floral symmetry, the ovaries are supposed to be formed by five carpels in Primulaceae (Dickson 1936; Clinkemaillie & Smets 1992) and Theophrastaceae (Cronquist 1981), and three to six carpels in Myrsinaceae (Mez 1902; Cronquist 1981). We infer that the gynoecium of *Myrsine laetevirens* is composed of five carpels because the ovary wall is supplied by 15 vascular bundles and five of them extend into the style, and also because the stigma is five-lobed.

M. laetevirens has hemianatropous, tenuinucellate ovules similar to those described in general for Myrsinaceae (Davis 1966; Johri *et al.* 1992), but they are unitegmic and not bitegmic such as those of *Ardisia*, *Embelia* and *Maesa* (Netolitzky 1926; Schürhoff 1926; Sankara Rao 1972; Philipson 1974; Corner 1976; Dahlgren 1980; Johri *et al.* 1992). Up to now, only two genera of the Primulales, *Aegiceras* of Myrsinaceae (Carey & Fraser 1956; Corner 1970; Philipson 1974) and *Cyclamen* of Primulaceae, had been reported as having unitegmic ovules (Anderberg & Ståhl 1995). According to Dahlgren (1975, 1980), Philipson (1974) and Sporne (1969), the number of integuments is a very important embryological feature from an evolutionary point of view. Bouman (1984) considers bitegmy as the primitive condition and recognizes different processes of unitegmization. He reports 16 dicotyledonous families, in which Myrsinaceae is not included, with both bitegmic and unitegmic species. In order to clarify the changeover from bitegmy to unitegmy and to elucidate the evolutionary path within the family, it becomes necessary to study the structure and development of the ovules in the remaining genera of Myrsinaceae.

The presence of an endothelium in Myrsinaceae was reported only in the ovules of *Maesa dubia* (Sankara Rao 1972). In *Myrsine laetevirens* there is no endothelium.

In *M. laetevirens*, the embryo sac development corresponds to the Polygonum type, being similar in this respect to *M. africana* (Dahlgren 1916). The same type of development has been found in the rest of the studied species of Myrsinaceae (Davis 1966; Johri *et al.* 1992).

The presence of a placenta with special characteristics is one of the most important finding of this study. During the anthesis the placenta occupies the locule almost entirely and partly surrounds the ovules. As a mucilage-secreting structure, the placenta develops grooves and canals which increase the secreting area. The mucilage keeps the bacterial population thriving. A placenta with similar characteristics has been described by Miller (1990) in *Ardisia*.

On the other hand, *Ardisia*, *Amblyanthus* and *Amblyanthopsis* are the genera of the Myrsinaceae having species with nodules in their leaves produced by a symbiosis with bacteria (Miehe 1911; De Jongh 1938; Lersten & Horner 1976; Lersten 1977; Gardner *et al.* 1981; Miller *et al.* 1983, 1984; Miller 1990). According to Miller (1990) the transmission of the bacteria in *Ardisia crispa*, from one host plant generation to the next, takes place within the ovary. Leaf nodules are absent in *M. laetevirens* but the sequence of events occurring inside the gynoecium is similar to that described by Miller in *A. crispa*. In this study we found some special features that have not been mentioned in *Ardisia*; for example, (i) at anthesis the mucilage was seen descending from the stylar canal to the substylar chamber of the ovary and (ii) the canals, which run through the placenta, allow free circulation of the mucilage towards the micropyles.

In *A. crispa* and probably in *M. laetevirens*, the mucilage with bacteria flows through the micropyle into the embryo sac, thus positioning the bacteria in a situation where it can be incorporated into the seed. In this way, a coordinated dispersal of the seeds

and the bacteria seems to take place. This phenomenon has parallels with coordinated dispersal of seeds and fungal pathogens (Popp 1951), seeds infected by viruses (Gibbs 1983), and even seeds of parasitic angiosperms with the seeds of their hosts (Atsatt 1965).

The bacteria found in *Ardisia* have been identified under several names (Miller 1990) and described as Gram-negative; they are gently curved rods of 2–5 μm but pleiomorphic in mature and older nodules. Studies carried out to detect nitrogen fixation in myrsinaceous plants with bacterial leaf nodules have all proved negative. However, *Ardisia* plants can not grow normally without the bacteria. All evidence to date shows that some kinds of plant regulators, probably cytokinins, are excreted by the bacteria (Miller 1990). Contrary to *Ardisia*, there are no leaf nodules in *Myrsine* and the bacteria are globular. Two basic questions remain unknown about the bacteria found in *M. laetevirens*: (i) the identity of the bacteria; and (ii) the benefits, if any, that the bacteria provide the host. We propose to take up these topics in further papers.

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