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POLLEN INSIGHTS INTO APOMICTIC AND SEXUAL *MICONIA* (MICONIEAE, MELASTOMATACEAE)

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The genus *Miconia*, Melastomataceae, has been highlighted for its high proportion of apomictic species, in which the occurrence of low pollen grain viability is commonly recorded. In this study, we compared aspects of pollen development and viability and ploidy level in a sexual species, *Miconia pepericarpa*, and an apomictic species, *Miconia fallax*, and investigated the possible causes of pollen grain sterility in apomictic species. Abnormal meiosis was observed only in apomictic *M. fallax* and leads to the formation of inviable pollen grains, often with little or no cytoplasmic content. Furthermore, the apomictic species is polyploid, and therefore, chromosomal imbalance would be expected. Symmetric mitotic division was found only in *M. fallax*, also resulting in inviable pollen grains, usually with cytoplasm content but without generative cell differentiation. At the end of development, the apomictic species also displays another difference when compared with the sexual species: the generative cell does not become fusiform but, rather, maintains a spherical shape. It is unknown whether the generative cell shape may interfere with its function.

Keywords: apomixis, pollen sterility, polyploidy, abnormal meiosis, symmetric mitosis.

Introduction

In Angiospermae, reproduction may involve the production and fusion of gametes (i.e., sexual reproduction) or may occur independently of these processes (i.e., asexual reproduction; Richards 1977; Nogler 1984; Karasawa et al. 2009). The asexual reproduction can occur through different strategies, including apomixis (Richards 1977; Nogler 1984; Karasawa et al. 2009), defined as a process of asexual reproduction by seeds, with the formation of a progeny of maternal origin, or simply cloning through seeds (Asker and Jerling 1992). Apomixis results originally from a deregulation, in time and space, of sexual development, which leads to a change in the fate of cells and omission of critical events in the sexual process (Koltunow and Grossniklaus 2003; Tucker and Koltunow 2009).

Apomictic processes have been described in ~33 families of Angiospermae (Carman 1997) and are markedly more frequent in some groups of plants, such as Asteraceae, Poaceae, Rosaceae, and Rutaceae (Richards 1977; Naumova 2008). In Melastomataceae, a tropical plant family, apomixis is also common, especially in tribe Miconieae, in which ~63% of the species are apomictic (Goldenberg and Shepherd 1998). If the data of the reproductive system in this family are representative and if the proportion of apomictic species is constant, the tribe Miconieae would be one of the largest apomictic complexes studied to date (Goldenberg and Shepherd 1998). Thus, the group offers a unique opportunity to discuss the apomictic process in a different scenario from what has been studied so far, with natural representatives from various habits, particularly tropical shrubs and trees (Goldenberg et al. 2008). Furthermore, unlike the majority of apomictic plants that presents pseudogamy (Mogie 1992; Horandl 2010), *Miconia* species show autonomous apomixis (Renner 1989; Goldenberg and Shepherd 1998; Goldenberg and Varassin 2001), because the production of viable seeds is independent of the presence of pollen as well as central cell fertilization.

Miconia is the largest genus within the Miconieae, with ~1050 species of the 1800 representatives of this tribe (Goldenberg et al. 2008; Penneys et al. 2010). There seems to be a close relationship between apomixis and low pollen viability in Miconieae species (Goldenberg and Shepherd 1998; Goldenberg and Varassin 2001). Apparently, low levels of viable pollen in this group are related to alterations during meiosis in hypothesized polyploid species (Goldenberg and Shepherd 1998; Melo et al. 2009; Cortez et al. 2012; Caetano et al. 2013).

In this context, the goal of this study was to compare aspects of the pollen development and viability and ploidy level in sexual and apomictic species of *Miconia*, whose reproductive systems were previously set (Goldenberg and Shepherd 1998) and confirmed by unpublished data regarding female development (A. P. S. Caetano, personal observation).

Material and Methods

Two species of *Miconia* were employed: *Miconia fallax* DC., an apomictic species, and *Miconia pepericarpa* Mart. ex DC., a sexual species. The vouchers were deposited into the her-

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barium at Universidade Estadual de Campinas (UEC) in São Paulo, Brazil, under the accession numbers 150450 and 150451, respectively. Samples were collected during 2008–2010 in natural areas located in cerrado vegetation in the municipality of Itirapina, São Paulo, Brazil (lat. 22°15′10″S, long. 47°49′22″W).

For the anatomical analysis, floral buds and flowers of five individuals from each species were fixed in a solution composed of 80 mL L^{-1} glutaraldehyde, 250 mL L^{-1} paraformaldehyde (16%), and 500 mL L^{-1} phosphate buffer (0.1 M, pH 6.8) for 24 h (modified from Karnovsky 1965). The samples were embedded in glycol methacrylate Historesin Leica. Cross sections between 1.0 and 3.0 μm thick were stained with 0.05% toluidine blue (CI 52040) in phosphate buffer, pH 6.8 (modified from Feder and O'Brien 1968).

To verify the deposition of callose wall during pollen development, some slides were treated with aniline blue (CI 42755; Kapil and Tiwari 1978) and observed using a fluorescence microscope equipped with a UV excitation filter.

Anthers of 19 floral buds at the meiotic stage from five individuals of *M. fallax* were crushed on slides with acetocarmine solution for the analysis of abnormal meiosis. A total of 1986 structures were analyzed and classified as tetrads (four microspores) or polyads (five to seven microspores).

Pollen viability was tested with DAB (3.3′ diaminobenzidine, Sigma Fast; Rodriguez-Riano and Dafni 2000). Twenty-six individuals were used for *M. fallax*, and six were used for *M. pepericarpa*. The difference in the number of individuals used was due to the habit and population structure of each species collected. *Miconia fallax* are shrubs up to 2 m in height and form a "dense population," whereas *M. pepericarpa* are shrubs or trees up to 5 m in height with scattered individuals in the study area. For each individual, all anthers of eight floral buds at preanthesis were analyzed. Each slide was prepared from a single floral bud. The first 100 pollen grains on each slide were counted and classified. Pollen grains were classified as viable when they acquired a dark brown coloration.

An estimation of chromosome number of the two species was performed by mitotic analysis of root tips. These materials were pretreated with 8-hydroxyquinoline 0.2 M at room temperature for 5 h, fixed in ethanol/glacial acetic acid (3 : 1 v/v) for 24 h at room temperature, and stored in a freezer. To prepare slides, the root tips were hydrolyzed in 5 N HCl at room temperature for 20 min, washed in distilled water to remove the solution, mounted on cover slips, and squashed in 45% acetic acid. The slides were frozen in liquid nitrogen to remove the cover slip. The preparations were stained with Giemsa following standard protocol (Guerra and Souza 2002).

All photographs were taken using an Olympus BX51 microscope coupled with a digital camera model DP71. From pollen viability and reproductive system data available in the literature, we made a graph comparing pollen viability between apomictic and sexual species of Melastomataceae.

Results

Pollen Development

Miconia fallax and *Miconia pepericarpa* have 10 and 8 stamens, respectively (fig. 1*A*, 1*B*), with bithecate and tetrasporangiate anthers (fig. 1*C*, 1*D*). The two microsporangia of each theca are separated by a septum formed by parenchyma cells that degenerate at anthesis and form a single locule in each theca (fig. 1*D*).

During microsporogenesis, the anther wall is composed of uniseriate epidermis, endothecium, a middle layer, and tapetum (fig. 1*E*). During anther development, the middle layer and tapetum degenerate, and only the epidermis and endothecium form the parietal layer of mature anther (fig. 1*F*). In anthers from open flowers, the endothecium cells, mainly at the apex of the anther, also degenerate.

In both species, sporogenous cells (fig. 2*A*) go through successive mitoses until differentiating into mother cells of microspores (fig. 2*B*). Just before the start of meiosis, the callose deposition in microspore mother cells occurs (fig. 2C), which isolates them from others. The cytokinesis is simultaneous (fig. 2*D*–2*I*), which leads to formation of tetrahedral tetrads (fig. 2*J*, 2*K*). A callose wall layer surrounds the microspores within the tetrads (fig. 2*L*).

Meiotic irregularities were observed in *M. fallax* as early migration of chromosomes in metaphase (fig. 2*E*) and laggard chromosomes in anaphase (fig. 2*F*, 2*H*). Normal tetrads (with four microspores of similar size; fig. 2*J*–2*L*), polyads of microspores (fig. 2*M*), and tetrads with microspores with micronuclei (fig. 2*K*) were found. The presence of polyads was observed in 39.33% of the analyzed structures.

The newly separated microspores have dense cytoplasm and central prominent nucleus (fig. 2*N*). The early stage of microgametogenesis is marked by two processes: microspore polarization, with migration of the nucleus from the center to the periphery of the microspore, and the formation of a large vacuole in the microspore (fig. 3*A*, 3*B*). These events culminate in the asymmetric microspore division, which gives rise to pollen grain, formed by vegetative and generative cells (fig. 3*C*). There is a clear dimorphism between these two cells. In this stage, the vegetative cell is larger and occupies a central position in the pollen grain, whereas the generative cell is smaller and occupies the parietal position (fig. 3C).

Shortly after mitosis, the generative cell detaches from the pollen wall, becomes spheroidal, and moves toward the center of the vegetative cell (fig. 3*C*–3*H*). At the end of development, the species show variation in the shape of the generative cell. In *M. fallax*, this cell always remains spherical, even in the mature anther, whereas in *M. pepericarpa*, immediately before the release of the pollen grain (fig. 3*E*), the generative cell becomes fusiform (fig. 3*G*, 3*H*). In both species, the pollen grains are shed in monads at the bicellular stage (fig. 3*E*, 3*G*).

In *M. fallax*, nuclear polarization was not observed in some microspores, which leads to symmetric division (fig. 3*I*). In this case, the pollen grain contains two similar cells and no dimorphism; the cells are the same size, have spherical nuclei, and have equal nuclear chromatin condensation (fig. 3*J*).

Inviable pollen grains are formed in both species but are formed in markedly larger proportions in the apomictic species, *M. fallax* (fig. 3*K*). In this species, there are pollen grains with deposition of exine and intine but completely empty in mature anther (fig. 3*K*, 3*L*). In some cases, even when the cytoplasm and the nucleus are present, the pollen grains are smaller, have cytoplasm that is far less dense, and have irregular shape (fig. 3*L*) when compared with viable pollen grains.

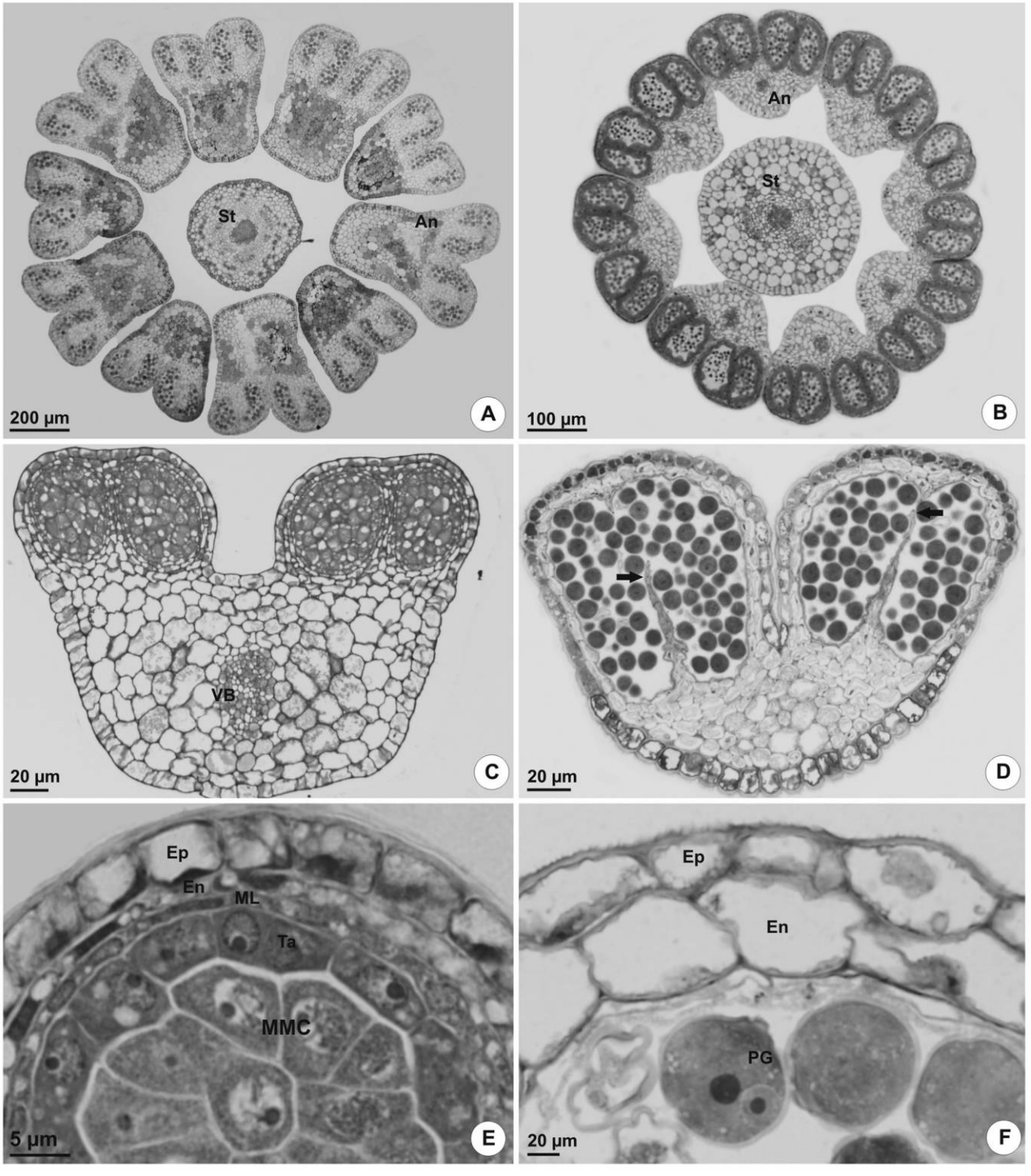

Fig. 1 General aspects of anther in sexual and apomictic *Miconia* species. *A*, *B*, Cross section of flower bud, showing all the anthers in *Miconia fallax* and *Miconia pepericarpa*, respectively. *C*, Anther in early stage of development in *M. pepericarpa*. *D*, Mature anther with intersporangial septum in degeneration (arrow) in *M. fallax*. *E*, Anther parietal layers in early stage of development in *M. pepericarpa*. *F*, Parietal layers of mature anther in *M. fallax*. An = anther, En = endothecium, Ep = epidermis, ML = middle layer, MMC = microspore mother cell, PG = pollen grain, St = style, Ta = tapetum, VB = vascular bundle.

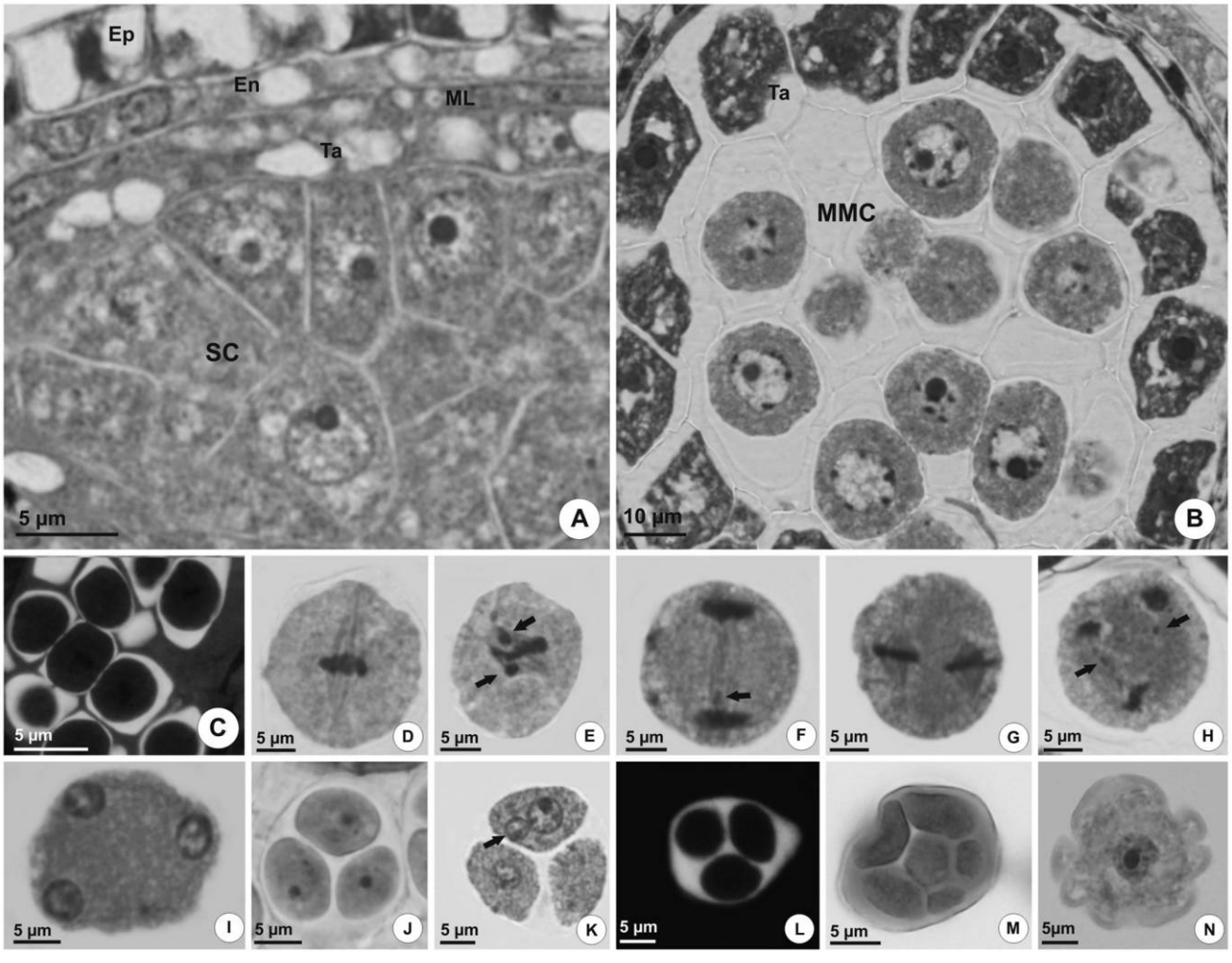

Fig. 2 Microsporogenesis in sexual and apomictic *Miconia* species. *A*, Anther parietal layers and sporogenous cells in *Miconia pepericarpa*. *B*, Microspore mother cells surrounded by tapetum in *Miconia fallax*. *C*, Callose surrounds the microspore mother cell in *M. fallax*. *D*, Metaphase in *M. fallax*. *E*, Metaphase I with precocious chromosomes ascension (arrows) in *M. fallax*. *F*, Anaphase I with laggards chromosomes (arrow) in *M. fallax*. *G*, Metaphase II in *M. fallax*. *H*, Anaphase II with laggards chromosomes in *M. fallax*. *I*, Telophase II in *M. fallax*. *J*, Microspore tetrad in *M. pepericarpa*. *K*, Microspore tetrad with micronuclei in one of the microspores (seta) in *M. fallax*. *L*, Microspore tetrad in *M. pepericarpa*. *M*, Microspore hexad resulting from irregular cytokinesis in *M. fallax*. *N*, Free microspore. En = endothecium, Ep = epidermis, ML = middle layer, MMC = microspore mother cell, SC = sporogenous cells, Ta = tapetum.

Ploidy Level

The chromosome number estimated for *M. fallax* was 2n = 58–68 (fig. 4*A*), and the chromosome number for *M. pepericarpa* was 2n = 34 (fig. 4*B*). In both species, the chromosomes were very small, not exceeding 1.0 μm in length.

Pollen Viability

Miconia fallax produces many fewer viable pollen grains than *M. pepericarpa* (fig. 5). In *M. fallax*, the average percentage of pollen grains that were viable was 29.16%, whereas in *M. pepericarpa*, the average was 73.73%. The compiled data from literature show that, in Melastomataceae, the apomictic species generally produce a smaller percentage of viable pollen grains compared with sexual species (fig. 6).

Discussion

Miconia fallax differed from *Miconia pepericarpa* in terms of pollen development and viability and ploidy level. These differences include the following: a lesser amount of viable pollen grains formed, abnormal meiosis and polyploidy, symmetric mitosis in some microspores, and the shape of generative cell in mature pollen grains of *M. fallax*.

Pollen viability of *M. fallax* and *M. pepericarpa* had been previously estimated using a test with acetocarmine (Goldenberg and Shepherd 1998). The value described for the apomictic *M. fallax* (39.5%) is close to the value found here (29.16%), which confirms that the species does indeed have some type of alteration that leads to the formation of a large proportion of inviable pollen grains, even in different years.

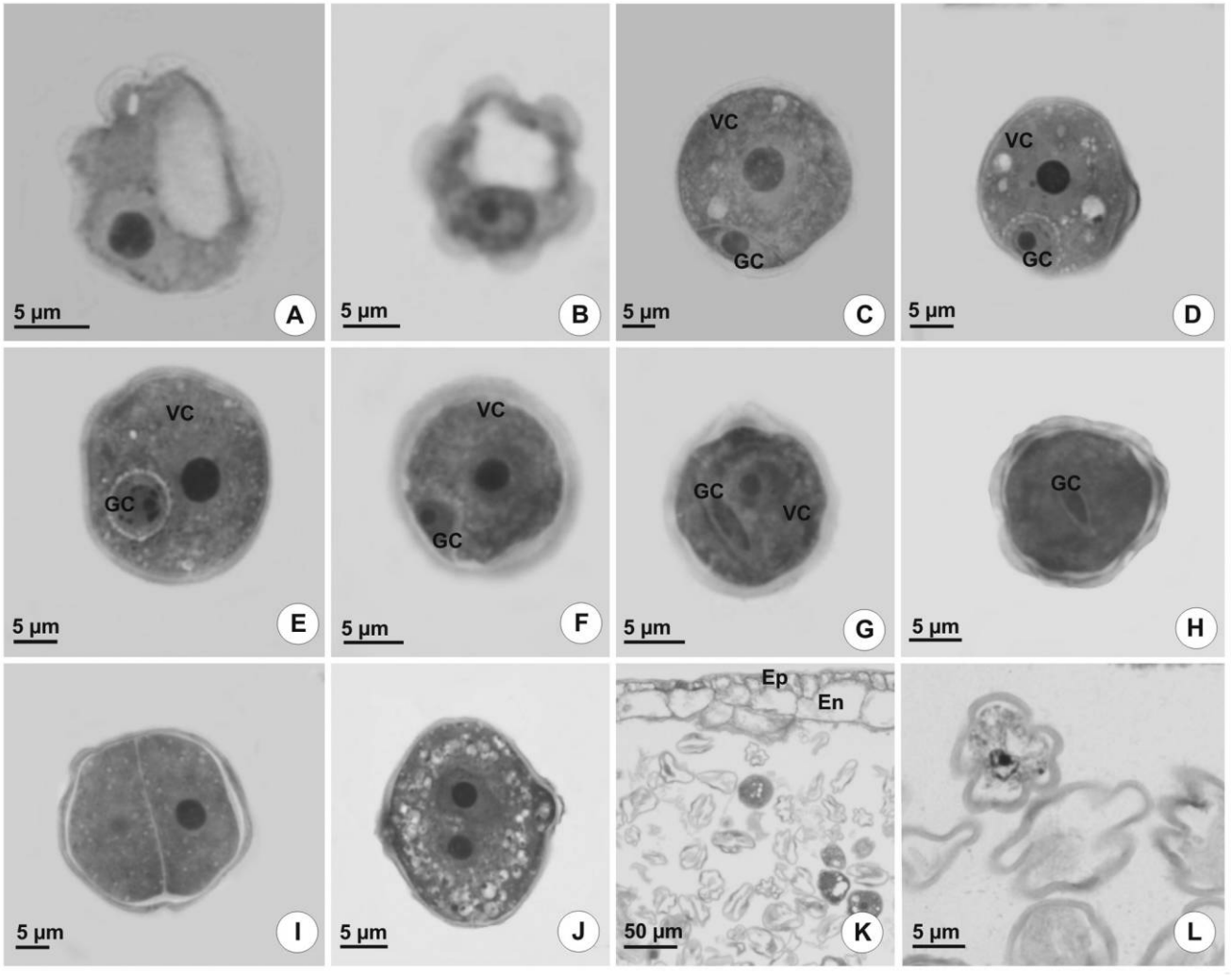

Fig. 3 Microgametogenesis in sexual and apomictic *Miconia* species. *A*, *B*, Vacuolated microspore in *Miconia fallax* and *Miconia pepericarpa*, respectively. *C–E*, Pollen grain in *M. fallax*. *F–H*, Pollen grain in *M. pepericarpa*. *I*, *J*, Abnormal pollen grain originated by symmetric mitosis. *K*, *L*, Abnormal pollen grains in *M. fallax*. En = endothecium, Ep = epidermis, GC = generative cell, VG = vegetative cell.

However, the data obtained here for *M. pepericarpa* show a higher proportion of viable pollen grains produced (73.73%) compared with the percentage described previously (59.8%; Goldenberg and Shepherd 1998). Pollen viability can be influenced by several factors that involve the environment and the plant itself, such as the nitrogen supply, humidity, temperature and time of anthesis, the conditions during microsporogenesis, the interspecific genetic variability, the metabolism of pollen (Dafni and Firmage 2000), and intraspecific natural variation, among other factors. All these factors, especially those dealing with the environment, contribute to the different proportions of viable grains produced in a given population over the years, which could explain the difference found in pollen viability data obtained previously (Goldenberg and Shepherd 1998) and in this study.

The pollen grain viability of several species of Melastomataceae was measured (fig. 6). A remarkable distinction between the average percentages of pollen grains formed by apomictic and sexual species was observed when the obtained data were compared. In the apomictic species, pollen viability ranged from ~0% to 60%, whereas in sexual species, these values were ~44%–98%. It has been suggested that there is a relationship between apomixis and inviable pollen (Asker and Jerling 1992; Mogie 1992), which is confirmed in Melastomataceae species (Renner 1989; Goldenberg and Shepherd 1998; Goldenberg and Varassin 2001; Melo et al. 2009; Cortez et al. 2012; Caetano et al. 2013; this study).

Pollen sterility in *M. fallax* is mostly associated with abnormal meiosis. Meiotic abnormalities have been described in some other apomictic species of Melastomataceae, such as *Miconia stenostachya*, a polyploid species with 80% inviable pollen grains, which has laggard chromosomes at anaphase I and chromosomal bridges at anaphase I and II (Goldenberg and Shepherd 1998; Cortez et al. 2012); *Clidemia bullosa* and *Clidemia capitellata*, which exhibit laggard chromosomes in anaphase I and tetrads with micronuclei, which lead to a high

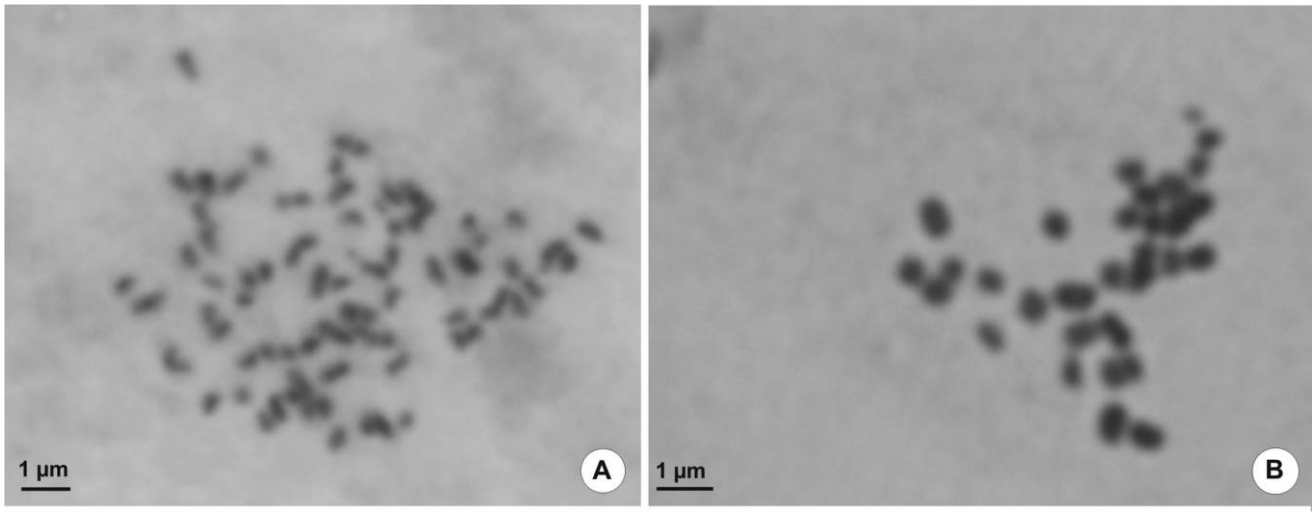

Fig. 4 Mitotic metaphase in sexual and apomictic *Miconia* species. *A*, *Miconia fallax* (2n = 68). *B*, *Miconia pepericarpa* (2n = 34).

pollen inviability (Melo et al. 2009); and *Miconia albicans*, a completely male sterile species, in which the meiosis is a rare event (Cortez et al. 2012). Low pollen viability due to meiotic irregularities is frequently reported in the literature, principally in economically important species (Pagliarini 2000; El Maâtaoui and Pichot 2001). Furthermore, these abnormalities and subsequent inviable pollen grains are common in polyploids, especially in allopolyploids, originated through interspecific hybridization and chromosome doubling (Stebbins 1950; den Nijs and Menken 1996).

Our data on ploidy level indicate that the apomictic population of *M. fallax* is polyploid, whereas that of *M. pepericarpa* is diploid. Assuming the base number of x = 17 for the tribe Miconieae (Solt and Wurdack 1980; Almeda and Chuang 1992; Almeda 1997), it is possible to infer that the *M. fallax* population studied is tetraploid (2n = 4x = ~68). Apomixis is often associated with polyploidy, and it is expected that almost all apomictic species are polyploids (Stebbins 1971; Asker and Jerling 1992; Carman 2007).

The polyploid origin of tribe Miconieae is certain (Almeda and Chuang 1992; Almeda 1997). It is believed that n = 17 originated by disploidy of autotetraploid with x = 9 (2x − 1) or by an ancient hybridization between species with haploid chromosome number 7 and 10 or 8 and 9, followed by chromosome doubling (Almeda and Chuang 1992). Moreover, in a few species of Miconieae, a secondary cycle of polyploidy gives rise to high chromosome numbers (Solt and Wurdack 1980; Almeda 1997), as in *Miconia mirabilis* (n = 68), *Miconia rubiginosa* (2n = 50; Solt and Wurdack 1980), and *M. stenostachya* (n = 26; Goldenberg and Shepherd 1998).

In addition to the alterations during meiosis, the symmetric first mitotic division within the microspore in *M. fallax* also contributes to pollen grain inviability, although this process is less commonly found. This behavior has been recorded for the apomictic species *M. stenostachya* (Cortez et al. 2012). It is known that the asymmetric pattern of the first mitotic division is essential for pollen grain functionality (Tanaka 1997; Twell et al. 1998) and can be considered a pattern in angiosperms (Batygina 2002). The symmetric division prevents generative cell differentiation, essential for normal pollen grain maturation (Tanaka 1997; Twell et al. 1998). Symmetric mitotic division in *Brachiaria decumbens* results in male sterility, and this anomaly can be explained by a mutation in cytoskeleton microtubule formation (Filho et al. 2003). A microtubule system is closely involved in nuclear migration, and division plane in asymmetric cell division and disturbances on microtubule could prevent generative cell differentiation (Tanaka 1997; Twell et al. 1998).

The generative cell morphology of *M. fallax* and *M. pepericarpa* contrasts markedly at the final stage of pollen development. In general, the shape of the generative cell varies during development, but it often becomes elongated at the end of this process (Batygina 2002). In the apomictic *M. stenostachya*, nonelongated generative cells were also observed (Cortez et al. 2012); however, apomictic Miconieae species can eventually produce normal pollen grains with elongated generative cells (A. P. S. Caetano, personal observation). Changes in the mi-

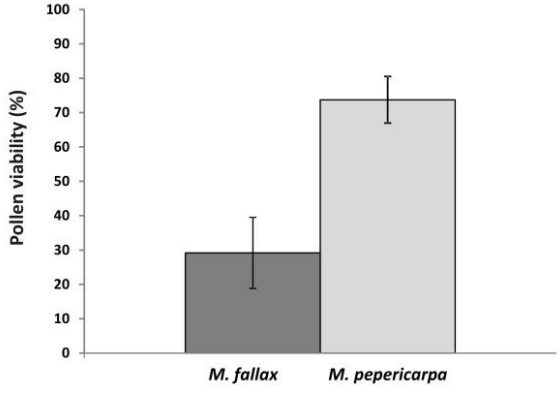

Fig. 5 The average percentage of viable pollen grains in apomictic and sexual *Miconia* species. The error bars indicate standard deviation.

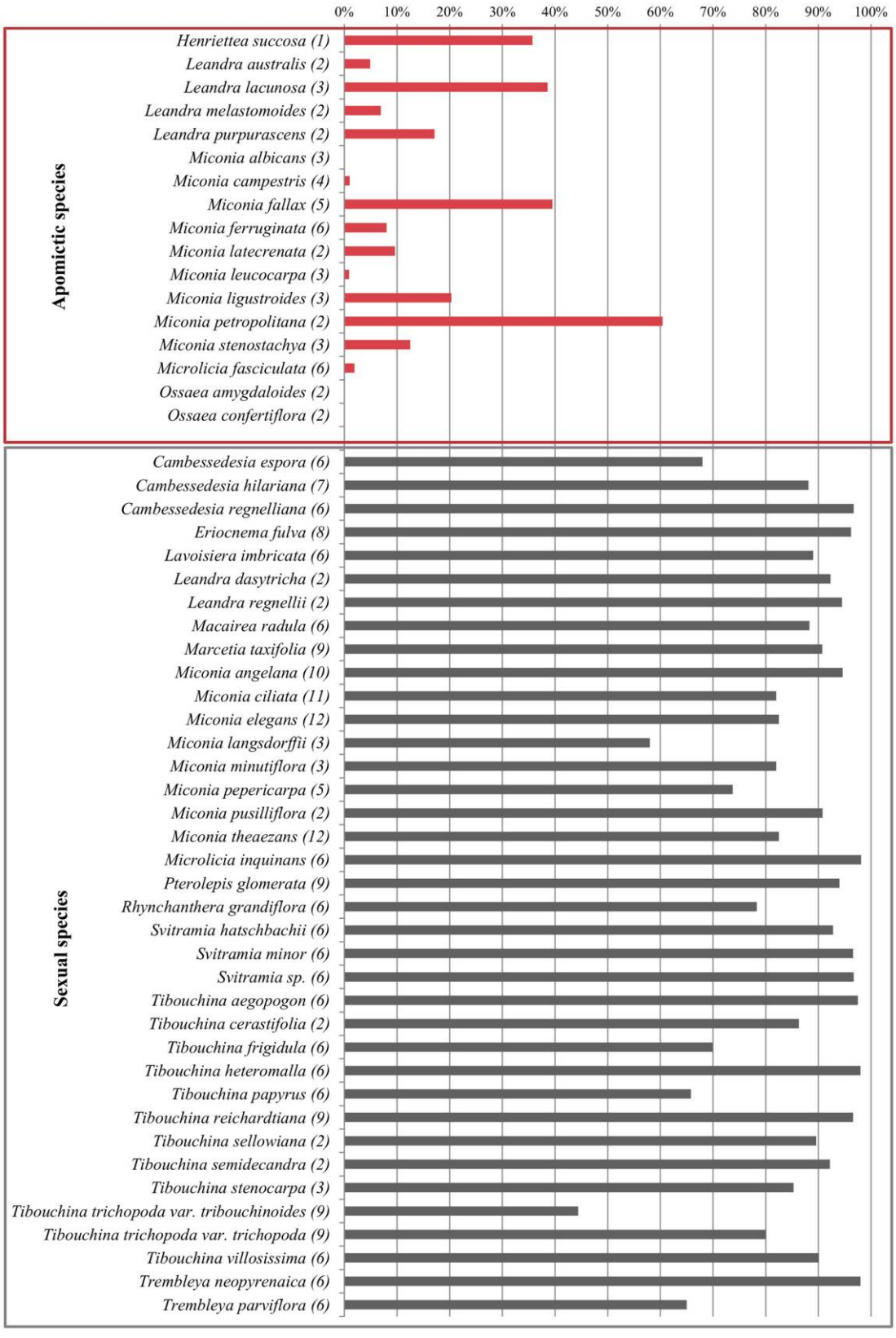

Fig. 6 Comparison of the average pollen viability between apomictic and sexual species of Melastomataceae. Source references: 1, Melo and Machado 1996; 2, Goldenberg and Varassin 2001; 3, Goldenberg and Shepherd 1998; 4, Renner 1989; 5, this study; 6, Santos et al. 2012; 7, Fracasso and Sazima 2004; 8, Andrade et al. 2007; 9, Pinheiro 1995; 10, Santos et al. 2010; 11, Melo and Machado 1998; 12, Borges 1991.

crotubule system could affect the elongation of the generative cell, which would explain the fact that the cell remains spherical in pollen grain dispersal. Studies have shown that damage in the microtubule system by a particular substance prevents the generative cell passing from the spherical to elliptical shape, which demonstrates the crucial role of microtubules in this process (Sanger and Jackson 1971). Moreover, this character state may be related to apomixis, which results from a deregulation of the sexual developmental programs (Koltunow and Grossniklaus 2003), leading to heterochronic phenotypes, which can be expressed in male function (Grimanelli et al. 2003).

In Melastomataceae, pollen grains are released in the bicellular stage (Tobe and Raven 1984; Medeiros and Morretes 1996; Medeiros and Ross 1996). Most angiosperms release bicellular pollen, and the sperm production division occurs within the pollen tube (Brewbaker 1967). The characteristic elongated shape of the generative cell enables it to travel down the pollen tube (Sanger and Jackson 1971; Palevitz and Tiezzi 1992). The spherical shape of the generative cell in *M. fallax* could hinder its migration through the pollen tube and prevent the occurrence of double fertilization. If this does occur, pollen grains, despite the normal appearance of the cytoplasm, could be classified as nonfunctional. Aspects of pollen germination and tube growth should be investigated to verify this hypothesis.

Although studies of the reproductive biology of Melastomataceae have reported low pollen viability in apomictic species (Renner 1989; Goldenberg and Shepherd 1998; Goldenberg and Varassin 2001), the possible causes of this phenomenon have been little investigated in this group, which represents a good model for apomixis studies. In addition to the expected meiotic abnormalities, our study demonstrates the symmetric division in mitosis as another cause related to pollen inviability. Furthermore, the generative cell shape in pollen grain could also interfere with the functionality of the pollen; however, this hypothesis should be further investigated. This study also indicates a polyploidy occurrence in apomictic species, as has been clearly confirmed in other groups (Carman 2007) but rarely explored in Melastomataceae.

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