Floral morpho-anatomy and reproductive ecology of *Spondias macrocarpa* engl. (Anacardiaceae), a vulnerable neotropical andromonoecious tree

Marília Claudiano Tavares[a], Elisabeth Dantas Tölke[b*], Carlos Eduardo Pereira Nunes[c], Sandra M. Carmello-Guerreiro[a]

[a] Instituto de Biologia, Universidade Estadual de Campinas. Departamento de Biologia Vegetal. CEP 13083-970, Campinas, São Paulo, Brasil.

[b] Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, CEP 05508-090, São Paulo, São Paulo, Brasil.

[c] University of Stirling Cottrel, Department of Natural and Environmental Sciences, Building FK9 4LA, Stirling Scotland, UK.

[*] Author for correspondence: elisabeth.tolke@gmail.com (ED Tölke)

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Abstract

The genus *Spondias* has a wide variety of sexual systems, with complete monoecious to polygamodioecious species. *Spondias macrocarpa* (Anacardiaceae) is an endemic species from Brazil and considered vulnerable due to massive deforestation, previously described as hermaphroditi[illegible] However, to our knowledge, no further details on the breeding system or morphological studies are available. Herewith we examine the floral morpho-anatomy and breeding system of this species to better understand the sexual function of different floral morphologies and the role of pollinators in its reproduction. We used light and scanning electron microscopy to study the morphology and anatomy of the flowers. Additionally, we carried out experiments on pollen viability, stigmatic receptivity, and controlled pollinations. We found both bisexual and male flowers in the same inflorescence, with the staminate flowers presenting cryptic unisexuality. Investment in bisexual flowers was higher than in male ones, although more than 70% of the inflorescence was made up of male flowers. Most male flowers are present in the proximal region of the inflorescence, and there is no temporal separation between male and female functions in bisexual flowers. We show that *S. macrocarpa* is andromonoecious with cryptic-monoecy and a self-incompatibility system. Under natural conditions, *S. macrocarpa* is probably a pollinator generalist, with small bees being its main floral visitors. High levels of pollen robbing and low levels of crossing by floral visitors may result in the observed low fruit set. Further studies on the pre- and post-pollination barriers and on the reproductive ecology of extant natural populations should elucidate the reason for such a low fruit set.

Keywords: floral anatomy, fruit tree, plant sexuality, reproductive biology, sexual system, tropical tree.

1. Introduction

The sexual strategies of plants vary in a wide spectrum, ranging from complete self-fertilization to obligate outcrossing (Goodwillie et al., 2005), and the diversity of sexual systems is considered crucial for the evolutionary success of angiosperms (Bawa and Beach, 1981; Cruden and Lloyd, 1995; Goodwillie et al., 2005). Such diversity in sexual systems is accompanied by a diversity in floral morphology that can ~~reflect~~ in the presence of different floral types in one or more individuals of a species (Sakai and Weller, 1999; Barret and Hough, 2013).

One of these sexual systems is ~~the~~ andromonoecy, in which individuals of a population have bisexual and staminate flowers, which may have a reduced, non-functional or even absent gynoecium (Cruden and Lloyd, 1995; Jong et al., 2008; Cardoso et al., 2018). Andromonoecy has evolved independently several times in many groups and is found in 33 families of angiosperms, only 2% of the angiosperms (Richards, 1997; Miller and Diggle, 2003, 2007). The production of unisexual male flowers favours reproduction, since it increases the number of male gametophytes and saves resources by limiting the development of a gynoecium to fewer flowers (Janzen, 1977; Lloyd, 1980; Oliveira and Maruyama, 2014; Cardoso et al., 2018). In this sense, andromonoecy is related mainly to species with relatively large and expensive fruits (Loyd, 1980; Oliveira and Maruyama, 2014), such as several species in the *Spondias* genus.

Spondias L. has about 18 species with Pantropical distribution, mainly in South America, Asia and Madagascar (Mitchell and Daly, 2015). It is represented in Brazil by at least nine species and occurrs naturally in almost all regions of the country, but some species are also cultivated (Silva-Luz and Pirani, 2015). *Spondias* has polysymmetric, pentamerous flowers, which may be unisexual or bisexual, most of the time the bisexual flowers are functionally male or female, depending on the species (Bawa, 1974; Croat, 1974; Lozano, 1986; Bachelier and Endress, 2009; Pell et al., 2011; Mitchell and Daly, 2015). A diversity of mating systems has been reported in this genus, including monoecy, andromonoecy, dioecy and polygamodioecy (Bawa, 1974; Croat, 1974; Nadia et al., 2007). Although floral biology has been correlated with sexual systems in some *Spondias* species (Bawa, 1974; Croat, 1974; Lozano, 1986; Nadia et al., 2007; Mitchell and Daly, 2015), the current understanding of the reproductive versatility and mating systems of the genus is limited. Despite being an economically important taxon (Mitchell and Daly, 2015), floral investigations and their link with the reproductive system are rare, due to the difficulty of conducting field studies with these plants, which are commonly tall trees. In this sense, *Spondias* can be used as a good model to clarify evolutionary trends regarding sexual systems.

Spondias macrocarpa Engl., is an endemic Brazilian tree species, which has a very limited distribution, occurring only in the Atlantic Forest areas of Northeast and Southeast of Brazil (Bahia, Minas Gerais, Espírito Santo and Rio de Janeiro) and is considered a vulnerable species, since many specimens of the known collection come from areas which are no longer forested (Mitchell and Daly, 2015; Silva-Luz and Pirani, 2015). To our knowledge, *S. macrocarpa* was never studied before in terms of its sexual system, despite being classified as hermaphroditic in original descriptions (Mitchell and Daly, 2015).

Indeed, the determination of the sexual system of this plant based solely on external flower morphology may lead to incorrect conclusions. However, this issue can be addressed by employing field experiments and anatomical studies to better understand the function of different floral morphologies (Mayer and Charlesworth, 1991). In this work, we use a comparative morpho-anatomical study of the reproductive structures of two floral morphs and their sexual functions to determine the sexual system of *S. macrocarpa*. Finally, we provide information about its floral ecology and mating system, evaluating whether the species is dependent on pollinators for reproduction.

2. Material and Methods

2.1 Plant material and study site

We carried out this study from October 2017 to December 2018 at the University of Campinas (Campus collection of living plants), located in Campinas (22.8184° S, 47.0647° W), São Paulo State, south-eastern Brazil. To sample the trees, we marked eight individuals to be studied, from a population made up of 13 individuals, all them at the reproductive stage. Voucher specimens were deposited in the UEC Herbarium (UEC 119562).

2.2 Morphology and anatomy of floral morphotypes

We evaluated the floral morphology of three individuals to test whether there were variations in the androecium and gynoecium that could indicate the presence of functionally unisexual flowers. Thus, we collected 20 flowers from 10 inflorescences of each individual, which were analysed either fresh or fixed (stored in 70% ethanol), with a hand lens (60X increase) or under a stereomicroscope (Leica M80).

To test if there was sexual dimorphism, we analysed the diameter and height of the flower, the length of the petals, the height of the stamens, and the diameter and height of the gynoecium of each floral morphotype (n = 15 flowers/floral morphotype/individual, from four individuals). We also evaluated the morphological differences of pollen grains between the different floral morphotypes under SEM (Scanning Electron Microscopy, see procedure below), measuring the polar and equatorial diameter, and the colpus length (n = 10 pollen grains/floral morphotype, from three individuals). To evaluate the floral dimorphism the values obtained were submitted to the ANOVA test, and to evaluate the differences between the pollen grains from the different floral morphotypes the results were submitted to the Student's T-test, both using R software (R Core Team, 2018).

For anatomical studies, flowers of different morphotypes were fixed in FAA (formaldehyde, acetic acid, 50% ethanol) for 24 h (Johansen, 1940). The material was then dehydrated in an ethanol series and embedded in hydroxyethyl methacrylate resin (Historesin® Leica), according to Gerrits and Smid (1983). Transverse and longitudinal 8 μm thick sections were obtained using a Microm HM340E rotary microtome and stained with 0.05% Toluidine blue in sodium acetate buffer with a 4.7 pH (O'Brien et al., 1964). All slides were mounted with water and the images captured with an Olympus DP71 digital camera coupled to an Olympus BX51 microscope. The flowers were also observed under SEM, the previously fixed samples were dehydrated in an ethyl series, critical point dried (Balzers CPD-030), and sputter coated with gold (Balzers SCD-050). Observations were carried out using a Jeol JSM 5800 LV scanning electron microscope at 10 kV.

2.3 Functionality of floral morphotypes

We analysed the number of pollen grains produced by the flower and the pollen viability of the different morphotypes. The number of pollen grains was determined following the methodology proposed by Kearns and Inouye (1993). Flower buds were collected in pre-anthesis (n = 10 flowers/morphotype, from five different individuals). Then, anthers were dissected (n = 10 anthers randomly chosen, five replications) and stored in Eppendorf tubes at a controlled temperature (27 ° C) for 24 hours for dehiscence and consequent release of pollen grains. After 24 hours 1.000 µL of 70% ethyl alcohol was added to the tubes and homogenized in a vortex shaker for 30 s. We removed an aliquot of 1.5 µL and counted the number of pollen grains in a sedgwick rafter, under a light microscope (Olympus BX51). We deduce the value found in the aliquot for the initial sample to calculate the number of pollen grains produced per flower.

To test the viability of pollen grains from the different floral morphotypes, two treatments were used. In the first treatment, we tested pollen viability with 2% acetic carmine (Radford et al., 1974) in 10 inflorescences from three individuals. To do this, we macerated five anthers of different flowers of the same inflorescence in a drop of acetic carmine. In each slide, we counted 100 pollen grains under a light microscope, separating them into viable and non-viable based on their color, shape and size (n = 30 slides for each floral morphotype) (Kearns and Inouye, 1993). The second test was performed through the germination of pollen grains in 10% sucrose solution (concentration determined by previous experiments); the germinated pollen was counted 24 hours after incubation (n = 30 slides for each floral morphotype). The pollen grains were considered germinated when the pollen tube length reached the pollen diameter (Dafni, 1992). We submitted the values obtained for the different morphotypes to the "Student" t test at $P < 0.05$ (R Core Team, 2018), after applying the arcsine transformation to the data of both treatments to meet the normal distribution premise.

Stigma receptivity was tested through the observation of pollen tube growth. Pistils were self- and cross-pollinated, and after 24 and 48 h (n = 5 flowers/individual, from three individuals) of manual pollination, they were collected and fixed in 50% FAA (formalin–acetic acid–alcohol) (Johansen, 1940). Pistils were cleared with NaOH 9N, stored at 60ºC for 20 min, and stained with blue aniline (Martin, 1959 modified). Then, using a fluorescent microscope (Olympus BX51), we observed the germination of pollen grains.

2.4 Characterization of inflorescences

The number of flowers per inflorescence was evaluated (n = 50 inflorescences among five individual trees, 10 inflorescences/individual), as well the distribution of the floral morphotypes in the inflorescences. The opening sequence of the flowers in the inflorescences and the longevity of floral morphotypes were also observed. To evaluate whether there were differences between the number of male and bisexual flowers per inflorescence, the data was submitted to the Mann-Whitney test using R software (R Core Team, 2018).

2.5 Floral visitors

Focal observations of flower visitors occurred from 5:30 am to 3:30 pm totalling 50 h of observation. Weather conditions were not constant during all the days of focal observations, due to cloudy and sunny periods, but never rain during the observation time. Whenever possible, the visitors were photographed and captured with insect nets to obtain additional, more accurate identifications as possible with the help of specialists and literature. For every visitor observed, the collected resource (pollen or nectar) and contact with the flowers' stigma were recorded. The relative frequency of each visitor was calculated for the 2 years of field work. Visitors that contacted the stigmas and anthers were considered pollinators (following Alves-dos-Santos et al., 2016).

2.6 Mating system

For the mating system treatments, we selected five individuals for each treatment of sexual reproductive systems, including a control group (n= 10 inflorescences/individual/treatment). The inflorescences containing flower buds were previously bagged, to avoid any contact with pollinators, for the following hand-pollination treatments: (1) cross-pollination—pollen was transferred for flowers of different individuals, (2) self-pollination (autogamy)—pollen was transferred within-flower, (3) self-pollination (geitonogamy)—pollen was transferred from one flower to another from the same individual, and (4) spontaneous self-pollination—buds remained bagged and untouched to check the formation of fruits. Also, unbagged floral buds were marked and observed in order to estimate the success of pollination in natural conditions and these flowers were used as a control group (Dafni, 1992; Dafni et al., 2005). The self-incompatibility index was calculated as the ratio of the percentage of developed fruits after self- and cross-pollination, using the value of 0.25 (Bullock, 1985; Oliveira and Gibbs, 2000) as maximum limit to self-incompatibility. Reproductive efficiency was also calculated as the ratio between the set of fruits after natural- and cross-pollination (Zapata and Arroyo, 1978).

3. Results

3.1 Morphology and anatomy of floral morphotypes

Floral and sexual characterization indicated that *Spondias macrocarpa* is andromonoecious (possesses bisexual and male flowers in the same individual and in the same inflorescence). No female flowers were found in our survey. Male and bisexual flowers have five distinct green sepals and five distinct white petals, a nectary disk with ten lobes, ten stamens distributed in two whorls with introrse anthers of longitudinal dehiscence, and a five-carpel hypogenous ovary (Fig. 1A–F). In male flowers, the ovary is undeveloped (Fig. 1B), whereas in bisexual flowers the ovary is well developed with five linear-transversal stigmas (Fig. 1 E–F). In both male and bisexual flowers, the nectary is intrastaminal, i.e., the nectary is cup-shaped, glabrous, surrounding the ovary (Fig. 1 A–B, D–E).

Male and bisexual flowers were significantly different in size considering all analyses ($P < 0.05$), thereby the bisexual flowers were larger than the male flowers, also having larger petals and gynoecium, and higher stamens ig. 2). The pollen grains of both floral morphotypes were similar in size and morphology. Pollen grains were then oblate, three-colporate, convex-triangular in polar view, showing perforate to microreticulate exine (Fig. 3). No significant differences for the studied variables, namely polar diameter, equatorial diameter and colpus length, were identified between the floral morphotypes ($P > 0.05$) (Table 1).

The anthers are bithecal and tetrasporangiate and the anther wall layers were similar in both floral morphotypes, made up of an epidermis and an unstratified endothecium (Fig. 4A–B). Well-developed fibrous thickenings were seen in endothecium cells (Fig. 4B). The dorsal side of the anther was broader than the ventral side, presenting a relatively broad and thick connective (Fig. 4A). Mature pollen grains are observed even before the anthesis (Fig. 4C). At anthesis, the anther opens along the stomium slit in both thecae (Fig. 4A). In bisexual flower ovary, styles and stigmatic papillae were well-developed (Figs. 1E–F and 4C–E). The ovary contained four anatropou rassinucellate, pachycalazal ovules (Fig. 4F–G). Male flowers showed a short rudimentary pistil, where styles and ovary were inconspicuous, containing abortive ovules, and the stigmatic papillae were undifferentiated (Figs 1B and 5A–C).

3.2 Functionality of floral morphotypes

The mean number of pollen grains was not significantly different comparing both floral morphotypes. No differences ~~either~~ were observed for the viability of pollen grains for the two treatments performed (Table 2). The test using acetic carmine showed that about 95% of the pollen grains were viable, while the sucrose solution test predicted that about 75% of the pollen grains were viable (Table 2), and in both morphotypes, the anthers were dehiscent as of the first hours of anthesis. According to the test of stigma receptivity, only the bisexual flowers had a functional gynoecium, with the pollen grains germinating in the stigma surface up to three days after anthesis in self- and cross-pollination (Fig. 6).

3.3 Characterization of inflorescences

Spondias macrocarpa exhibited only one flowering episode per year, concentrated from October to December, with the highest values of flower intensity and activity (flowering peak) mainly during the first month. The flowers of *S. macrocarpa* were organized in panicles, where the male flowers were located mainly at the base of the inflorescence, decreasing towards the apex, while the opposite occurred with bisexual flowers (Fig. 7). No pattern in the sequence of opening flowers were observed, bisexual flowers presented approximately five days of longevity, while the male flowers presented only three days. The inflorescences produced between 70 to 1.100 flowers, 26.56% of which were bisexual and 73.44% were male (Mann-Whitney, U = 212; $P < 0.05$).

3.4 Floral visitors

The flowers of *Spondias macrocarpa* were visited by 15 insect species (Table 3). Apidae bees were the most frequent visitors, accounting for 65% of all the visits. The most frequent floral visitors were the eusocial stingless bees (Meliponini), *Trigona spinipes* (34.64% of the visits), and *Tetragonisca angustula* (28.39%), followed by invasive *Apis mellifera* (Apini, 13.54%). Most pollinator visits occurred during the morning from 5:30 AM to 10:00 AM, with the peak occurring between 7:00 AM and 09:00 AM. The flowers had a sweet smell and most of the visitors foraged looking for both nectar and pollen, except *Tetragonisca angustula*, flies and beetles, which collected only nectar. Due to the small size of flowers, all visitors ended up touching stigmas or anthers, thus being considered pollinators (Fig. 8).

3.5 Mating system

Spondias macrocarpa Engl. is a self-incompatible species with different pollination treatment results, no fruits developed in flowers bagged for autonomous and hand self-pollinated bisexual flowers (Table 4). The self-incompatibility index was 0.11 for spontaneous self-pollination, 0.11 for geitonogamy using pollen grains from male flowers, and 0.16 for geitonogamy using pollen grains from bisexual flowers. The largest fruiting rates were registered for cross-pollination experiments, using male and bisexual flowers, there were no statistical differences between these two treatments ($X^2 = 0.05 = 4.8786$; df 2; $P > 0.05$). The fruit set with hand cross-pollinated flowers was also higher than those obtained from natural conditions (for pollen coming from male flowers: $X^2 = 0.05 = 85.283$; df 2; $P < 0.001$; for pollen coming from bisexual flowers: $X^2 = 0.05 = 53.064$; df 2; $P < 0.001$). The reproductive efficiency calculated was 0.32 for cross-pollinated flowers using pollen grains from male flowers, and 0.46 for cross-pollinated flowers using pollen grains from bisexual flowers.

4. Discussion

4.1 Andromonoecy in *Spondias macrocarpa* in comparison to other Anacardiaceae and Sapindales

Our study showed that *Spondias macrocarpa* is andromonoecious. The most obvious difference between bisexual and male flowers is in pistil length, but differences in size of other floral organs were also found, indicating that the investment is higher in bisexual flowers.

Andromonoecy has been described for other species of *Spondias*, such as *S. tuberosa* and *S. mombin* (Nadia et al., 2007; Ramos, 2009; Carneiro and Martins, 2012). Similar findings are also documented for other species of Anacardiaceae. For example, in *Mangifera indica* and *Anacardium occidentale* bisexual and male flowers are found in the same inflorescence and also have a reduced gynoecium in male flowers (Moncur and Wait, 1986; Barfod, 1988; Wannan and Quinn, 1991; Tölke and Demarco, 2020). Many times, however, the morphological differences between the floral morphotypes are inconspicuous, therefore they are referred to as cryptic monoecious or dioecious (Anderson et al., 2015; Tölke et al., 2015). Due to this impossibility of visually distinguishing different floral morphotypes and their function, not only in Anacardiaceae species, but also in the Sapindales as a whole, we believe that in many species of the order, the sexual system needs to be revaluated (e.g., Alves et al., 2017). In Sapindaceae and Meliaceae, for instance, although some species apparently have male and bisexual flowers, anthers of the bisexual morphotype are empty or have unfertile pollen grains. Sometimes the anthers may not even release pollen grains, remaining indehiscent (Gouvêa et al., 2008; Solís et al., 2010; Rosado et al., 2018; Avalos et al., 2019; Zhou et al., 2019).

In species where the unisexual flowers have the rudiments of the nonfunctional organ, individuals may modify the frequency of the different floral morphotypes according to the available resources, which represents an innovation when compared to species where flowers bear no vestigial sexual organs (Primack and Lloyd, 1980; Solomon, 1986; Diggle, 1991; Vallejo-Marin and Rausher, 2007). In *Spondias mombin*, for example, individuals occurring in the State of Pará, Brazil, have been described as andromonoecious (Ramos, 2009). In contrast, this same species occurring in the State of São Paulo presented only bisexual flowers (Luz, 2011).

According to our results, all flowers of *S. macrocarpa* have functional pollen grains and release them at anthesis, confirming their male functionality. We also confirmed that there is not temporal separation between the male and female phases, demonstrated by the stigma receptivity test. Usually, Neotropical species of *Spondias* are described as protandrous (Mitchell and Daly, 2015), in which flowers present dehiscent anthers, while the gynoecium is still not completely developed, or the gynoecium is completely developed and anthers lack pollen grains. Thus, we reveal that the reproductive behaviour of *Spondias* is more complex and variable among species than previously known. In *S. macrocarpa,* the gynoecium of bisexual flowers is already mature at anthesis, and all the five locules yield a fertile ovule. This feature is not usual for *Spondias* and other phylogenetically close genera of Anacardiaceae, where the gynoecia generally have only one fertile ovule, no matter the number of carpels (Wannan and Quinn, 1991; Bachelier and Endress, 2009; Mitchell and Daly, 2015). Other species of the genus, such as *S. dulcis*, *S. purpurea* and *S. tuberosa*, all have a gynoecium with five carpels, five locules and only one fertile ovule (Bachelier and Endress, 2009). The evolution of investment in the formation of a single seed still needs to be explored in future works within Anacardiaceae.

4.2 The role of the inflorescence architecture in the pollination

Inflorescence architecture may have direct physical effects on the rate and movement of pollen in a plant population, the position of the different morphotypes in the inflorescence may affect the reproduction and the fruit set due to the behaviour of floral visitors (Wyatt, 1982). In *Spondias macrocarpa*, most of the male flowers are arranged at the base of the inflorescence, while the bisexual are located in the middle and at the apex, the same arrangement was observed in other species of the genus, such as in *S. tuberosa* (Nadia et al., 2007). According to Richards (1997), monoecious plants tend to have male flowers in a relatively proximal position on the plant, whereas female or bisexual flowers tend to be in a more distal position, corroborating our observations.

The main floral visitors of *S. macrocarpa* are bees, and according to Benham (1969) they generally move from the base to the apex of the inflorescence. In this process, the bees carry the pollen grains from the male flowers, located at the base, and move towards the bisexual flowers, facilitating the deposition of pollen on the stigmas of bisexual flowers (Benham 1969). *Spondias macrocarpa* has a generalist pollination system and 73.44% of its flowers are male. Species which presents a generalist pollination system have some disadvantages, since pollen can be used as a food resource by visitors, leaving less pollen available for reproduction (Pinheiro et al., 2014). This phenomenon is known as the “pollen dilemma” and is common among angiosperms (Westerkamp, 1997). Thus, the great quantity of male flowers in *Spondias macrocarpa* may be seen as a strategy to compensate for the pollen lost during the visits of insects.

The higher longevity of bisexual flowers in *S. macrocarpa* is unusual for monoecious plants (Richards, 1997). However, it is also observed in *S. tuberosa* and *S. mombin* (Nadia et al., 2007; Ramos, 2009), and may be explained by the fact that a greater exposure implies in a higher chance of these flowers being pollinated. Similar results were also documented by Primack (1980, 1985) for andromonoecious species, confirming that male flowers have a shorter longevity due their function of attraction and dispersion of pollen grains. Despite that, other elements may have influence in gender expression of plants~~,~~ Robbertse et al. (2011) showed that temperature and/or day length correlate closely with and may play an important role in the sex expression of the trees.

4.3 Floral visitors

Bees are considered the main pollinators of *Spondias macrocarpa*, which is quite ordinary among tropical species. Bees, in general, stand out both in abundance and in richness of species, most of them feed exclusively on floral resources (pollen grains, nectar and oil), and the search for food is one of the main reasons for visiting flowers (Agostini et al. 2014, Pinheiro et al.

2014). Thus, the high production of pollen, together with the production of nectar, contributes to the attraction of bees in *S. macrocarpa*. There are similar records for other species of *Spondias* and Anacardiaceae as a whole, where the main pollinators are also bees, although these plants are often generalists (Mitchell and Mori 1987, Lenza and Oliveira 2005, Nadia et al. 2007, Senchina and Summerville 2007, Ramos 2009, Almeida et al. 2011, Carneiro and Martins 2012, Fernandes et al. 2012).

The floral visitors who contributed with the largest number of visits were *Trigona spinipes* (34.64%), *Tetragonisca angustula* (28.39%) and *Apis mellifera* (13.54%). These generalist bees were also the most frequent in a study conducted by Agostini and Sazima (2003) about the relationship between ornamental plants and resources for bees in the same area where we carried out our study, reinforcing that regarding the pollination system *S. macrocarpa* and other Anacardiaceae species are most likely generalists. Visits of exotic bees like *A. mellifera* and *Trigona*, are known to interfere in the relationships between plants and their native pollinators, decreasing reproductive success in plants visited by them (Boiça-Jr. et al. 2004). However, in a study with *S. tuberosa,* the visits of those bees seemed to be beneficial (Almeida et al. 2011). More experiments should be carried out, in order to study the interference of exotic bees on the reproductive success of *S. macrocarpa*.

Another remarkable result here was the visits of the Scarabaeidae beetles of the genus *Macrodactylus*, which use the inflorescences of *S. macrocarpa* as mating sites. During their visits, they touch the reproductive organs of the flowers, attaching pollen grains on their head, chest, legs and mouth parts. In addition, these insects showed apparent mobility between different trees, thus being able to transfer pollen from one individual to another. According to Paulino-Neto (2014), 47% of the 34 plant families reviewed by Bernhardt (2000), present the Scarabaeidae beetles as exclusive or partial floral visitors. Therefore, these visitors are also considered as effective pollinators of *S. macrocarpa*.

4.4 Low fruit set

Our results suggest that the markedly low fruit initiation is the most restrictive step in fruit production for *Spondias macrocarpa*. Additional hand pollination significantly increased the fruit set compared with the control plants, demonstrating that *S. macrocarpa* suffer from pollinator limitation under the local conditions (Primack and Lloyd, 1980; Ashman et al., 2004; Reuther and Claßen-Bockhoff, 2013). We demonstrated here that *S. macrocarpa* is self-incompatible. Therefore, the reproduction occurs only by cross-pollination, which makes the pollinators essential for its reproductive success. However, even in the cross-pollination experiments, the low reproductive efficiency of the species was demonstrated, which may be linked to a low efficiency of the pollinators in the studied area (Bawa, 1977; Zapata and Arroyo, 1978), since the population is located out of its natural habitat.

In *S. macrocarpa,* the pollinators may promote a high rate of geitonogamy, contributing to a low fruit production under natural conditions. The low efficiency of natural pollination may also be associated with pollen robbers who take advantage of the resources offered by the species, but are not able to promote effective pollination. In addition, the low mobility of pollinators among different individuals of this self-incompatible species may also play an important role in limiting the fruit set. Studies with tropical trees have shown that cross-pollination is an important reproductive strategy, mainly because it promotes genetic variability and prevents inbreeding depression (Zapata and Arroyo, 1978; Sunnichan et al., 2004; Lenza and Oliveira, 2005). We here suggest that the low production of fruits, and also the occurrence of fruit abortion (Table 4) may be related to a low genetic variability of the studied population (Dubash and Fenster, 2000; Soares and Morellato 2018). Studies with other populations of *S. macrocarpa* from different areas are needed to confirm our hypothesis.

5. Conclusions

This study shows that *Spondias macrocarpa* is an andromonoecious species with cryptic-monoecy and self-incompatibility system. This species has bisexual and male functional flowers, where the male flowers still have an undeveloped pistil containing an abortive ovule. *Spondias macrocarpa* has more bisexual than male flowers, and although produces a high number of flowers per inflorescence, it has an extremely low fruit set. Further studies focusing on the pre- and post-pollination barriers should be carried out, in order to clarify the reason for this. For now, we hypothesize that high levels of pollen robbing may be a key factor in the low fruit set, since exotic bees like *A. mellifera* and *Trigona* are the main visitors of this species. The diversity of mating systems in *Spondias* make this genus an interesting model to test ecological and evolutionary questions about such traits.

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Marília Claudiano Tavares: Methodology, Validation, Investigation, Resources, Writing - Original Draft, Writing -Review & Editing, Visualization. **Elisabeth Dantas Tölke:** Conceptualization, Methodology, Writing -Original Draft, Writing -Review & Editing. **Carlos Eduardo Pereira Nunes:** Conceptualization, Methodology, Investigation, Methodology, Writing -Review & Editing. **Sandra Maria Carmello-Guerreiro:** Conceptualization, Resources, Writing -Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Mean and standard deviation of the pollen grains of bisexual and male flowers of *Spondias macrocarpa* Engl. P/E = ratio between polar and equatorial diameter

Floral morphotype/ variables	Polar diameter	Equatorial diameter	Colpus length	P/E
Bisexual	25.91 ± 1.74	42.51 ± 2.44	29.65 ± 2.36	0.60
Male	26.52 ± 1.87	41.69 ± 1.52	28.38 ± 3.33	0.64

No statistical significance was detected in any of the measured variables ($P > 0.05$). All measures are expressed in mean ± standard deviation (µm).

Table 2. Number (n) and viability (%) of pollen grains of bisexual and male flowers of *Spondias macrocarpa* Engl.

	Male flowers	Bisexual flowers
Number of pollen grains/anther	3046± 691	2337 ± 374
Number of pollen grains/flower	30462 ± 6913	23378 ± 3744
Viability: 2% acetic carmine	96.50 ± 1.63	95.77 ± 2.35
Viability: 10% sucrose solution	73.00 ± 8.70	77.67 ± 7.45

No statistical significance was detected in any of the variables ($P > 0.05$). All results are expressed in mean ± standard deviation.

Table 3. Relative frequency of floral visitors of *Spondias macrocarpa* Engl., the resource they were collecting (N: nectar and/or P: pollen), and flowering stage (O: outset, P: peak, F: final phase)

Flower visitors	Relative frequency (%)	Resource	Flowering Stage
Hymenoptera			
Hymenopera sp. 1	1.56	N	O/P
Apidae			
Apidae sp. 1	0.26	N/P	O/P/F
Apidae sp. 2	0.78	N/P	O/P/F
Apidae sp. 3	0.52	N/P	O/P/F
Meliponini			
Apis mellifera (Linnaeus, 1758)	13.54	N/P	O/P
Nannotrigona testaceicornis (Lepeletier, 1836)	8.33	N/P	P/F
Plebeia sp. 1	1.56	N/P	P/F
Plebeia sp. 2	1.56	N/P	P/F
Trigona spinipes (Fabricius, 1793)	34.64	N/P	O/P/F
Meliponini sp. 1	1.04	N/P	O/P/F
Meliponini sp. 2	3.39	N/P	O/P/F
Trigonini			
Tetragonisca angustula (Latreille, 1811)	28.39	N	O/P/F
Diptera			
Diptera sp. 1	0.26	N	O/P
Syrphidae sp. 1	1.04	N	O/P
Coleoptera			
Scarabaeidae			
Macrodactylus sp. 1	3.13	N	F

Table 4. Mating system of *Spondias macrocarpa* Engl. Percentage of fruits (number of fruits/number of pollinated flowers).

Pollination treatment	Initial fruits	Final fruits
	% fruits (n fruits/ n pollinated flowers)	
spontaneous self-pollination	0.41 (3/729)	0.41 (3/729)
hand self-pollination (autogamy)	0 (0/412)	0 (0/412)
hand self-pollination (geitonogamy using pollen grains from male flowers)	0.85 (3/352)	0.57 (2/352)
hand self-pollination (geitonogamy using pollen grains from bisexual flowers)	0 (0/1223)	0 (0/1223)
spontaneous cross-pollination (control)	3.31 (72/2173)	1.61 (35/2173)
hand cross-pollination (using pollen grains from male flowers)	12.48 (75/601)	4.99 (30/601)
hand cross-pollination (using pollen grains from bisexual flowers)	9.31 (93/999)	3.50 (35/999)

Figure captions

Fig. 1 Floral morphotypes of *Spondias macrocarpa* Engl. (A–C) Male flower. (D–F) Bisexual flower. General aspect of gynecence and nectary in scanning electron microscopy. (C) Anther dehiscence. (F) Style and linear-transversal stigma. Bars: 200 µm (C and F), 500 µm (B and E), 0.2 cm (A and D). Gy, gynoecium; Ne, nectary; Sg, stigma.

Fig. 2 Sexual dimorphism in floral morphotypes of *Spondias macrocarpa* Engl. The boxplot represents the median and quartiles while the horizontal line represents minimum and maximum values of the data range, circles represent outliers. Different letters indicate statistical significance ($P < 0.05$). All measures are expressed in millimetres.

Fig. 3 Pollen grains SEM of *Spondias macrocarpa* Engl. Since the pollen grains are morphologically similar, only the pollen grains of male flowers are shown. (A) Equatorial view. (B) Polar view. Bars: 10 µm.

Fig. 4 Anatomy of bisexual flowers of *Spondias macrocarpa* Engl. (A) Anthers are bithecal and tetrasporangiate. (B) Anther wall, constituted by an epidermis and an unstratified endothecium. (C) Floral bud in cross section, the five styles are showed. (D) Ovary in cross section containing five fertile ovules. (E) Longitudinal section of stigma presenting papillae. (F) Longitudinal and (G) cross section of the anatropous ovule. Bars: 40 µm (E, G), 50 µm (F), 100 µm (B), 200 µm (A, D), 400 µm (C). Co, connective; Ep, epidermis; En, endothecium; Ft, fibrous thickenings; Ov, ovule; Sp, stigmatic papillae; St, stomium; Sy, style.

Fig. 5 Anatomy of male flowers of *Spondias macrocarpa* Engl. (A) Floral bud in cross section, the five styles are showed. (B) Longitudinal and (C) cross section of the ovary containing abortive ovules. Bars: 100 µm (B, C), 400 µm (A). Ov, ovule; Sy, style.

Fig. 6 Stigma receptivity in bisexual flowers of *Spondias macrocarpa* Engl. (A) Control. (B, D, F and H) Pollen from bisexual flowers. (C, E, G and I) Pollen from male flowers. (B and C) Flower in anthesis. (D and E) Flower 24 h after anthesis. (F and G) Flower 48 h after anthesis. (H and I) = Flower 72 h after anthesis. Bars: 50 µm.

Fig. 7 Schematic drawing of the inflorescence of *Spondias macrocarpa* Engl. showing the distribution of male and bisexual flowers.

Fig. 8 Floral visitors of *Spondias macrocarpa* Engl. (A) *Nannotrigona testaceicornis*. (B) *Trigona spinipes*. (C) Syrphidae sp. 1. (D) *Plebeia* sp. 1. (E) *Tetragonisca angustula*. (F) *Nannotrigona testaceicornis*. (G) *Macrodactylus* sp. 1. (H) *Scaptotrigona depilis*.

Figure1

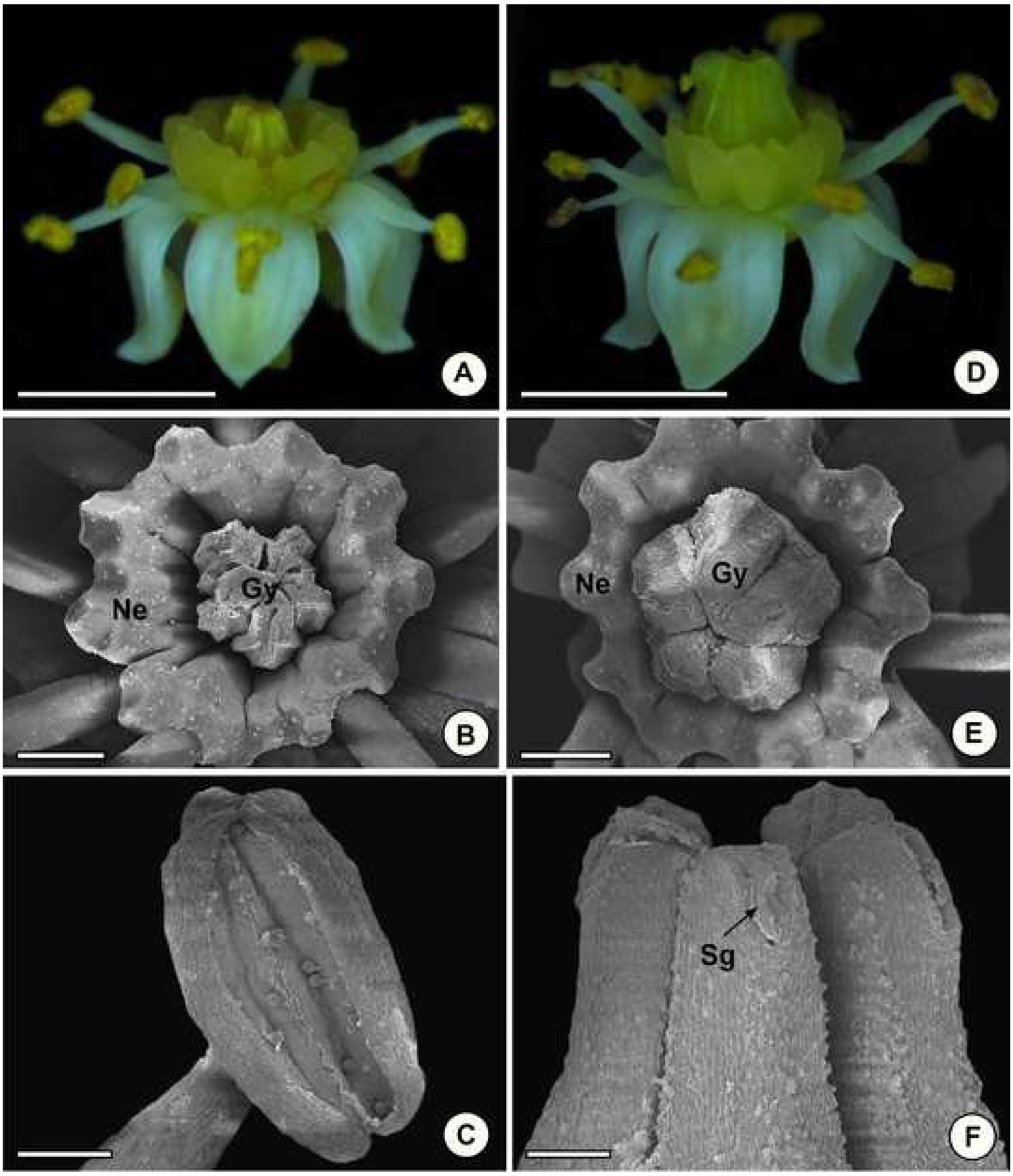

Figure2

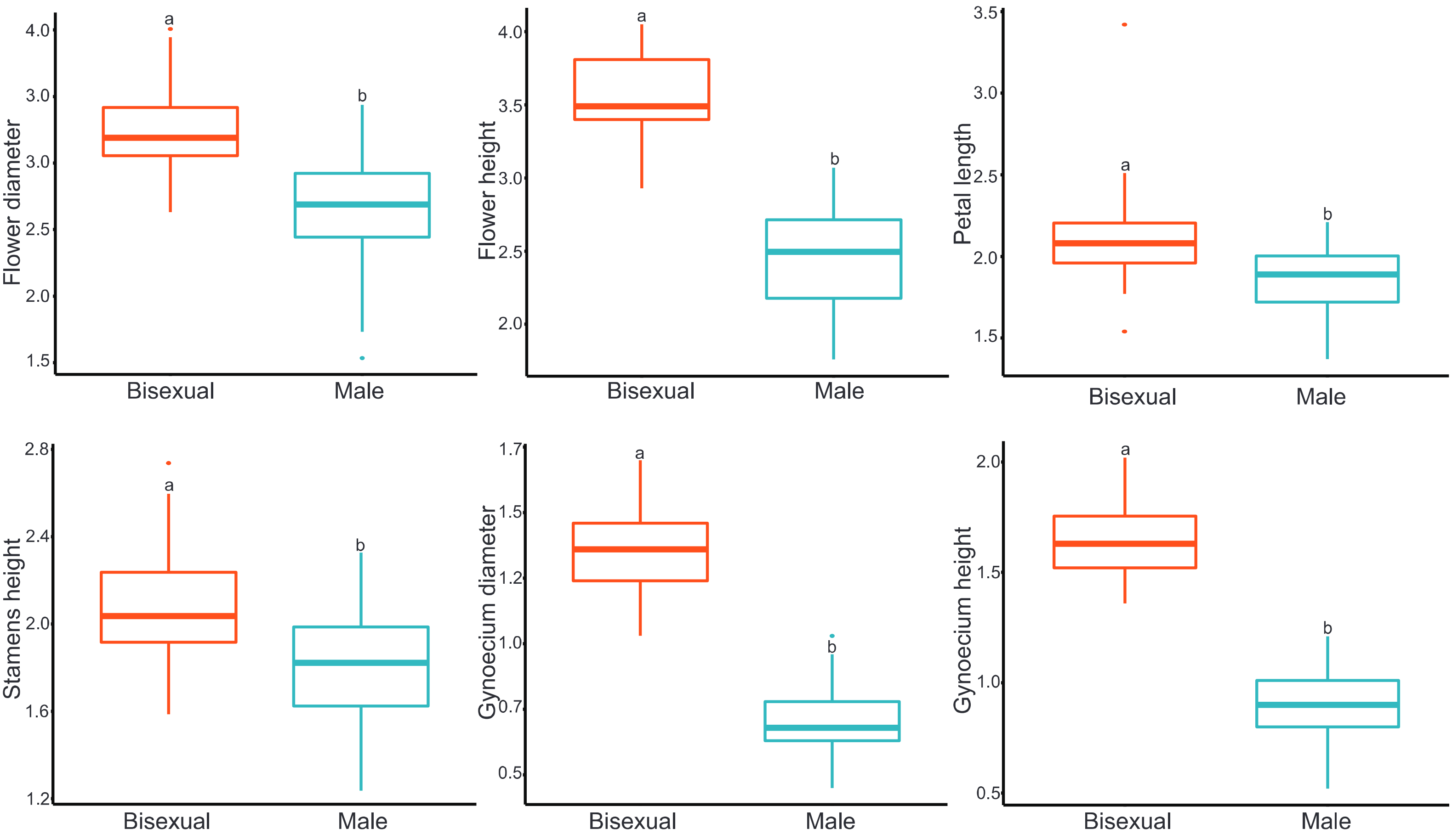
Flower diameter
4.0
3.0
3.0
2.5
2.0
1.5
a
b
Bisexual
Male
Flower height
4.0
3.5
3.0
2.5
2.0
a
b
Bisexual
Male
Petal length
3.5
3.0
2.5
2.0
1.5
a
b
Bisexual
Male
Stamens height
2.8
2.4
2.0
1.6
1.2
a
b
Bisexual
Male
Gynoecium diameter
1.7
1.5
1.2
1.0
0.7
0.5
a
b
Bisexual
Male
Gynoecium height
2.0
1.5
1.0
0.5
a
b
Bisexual
Male

Figure3

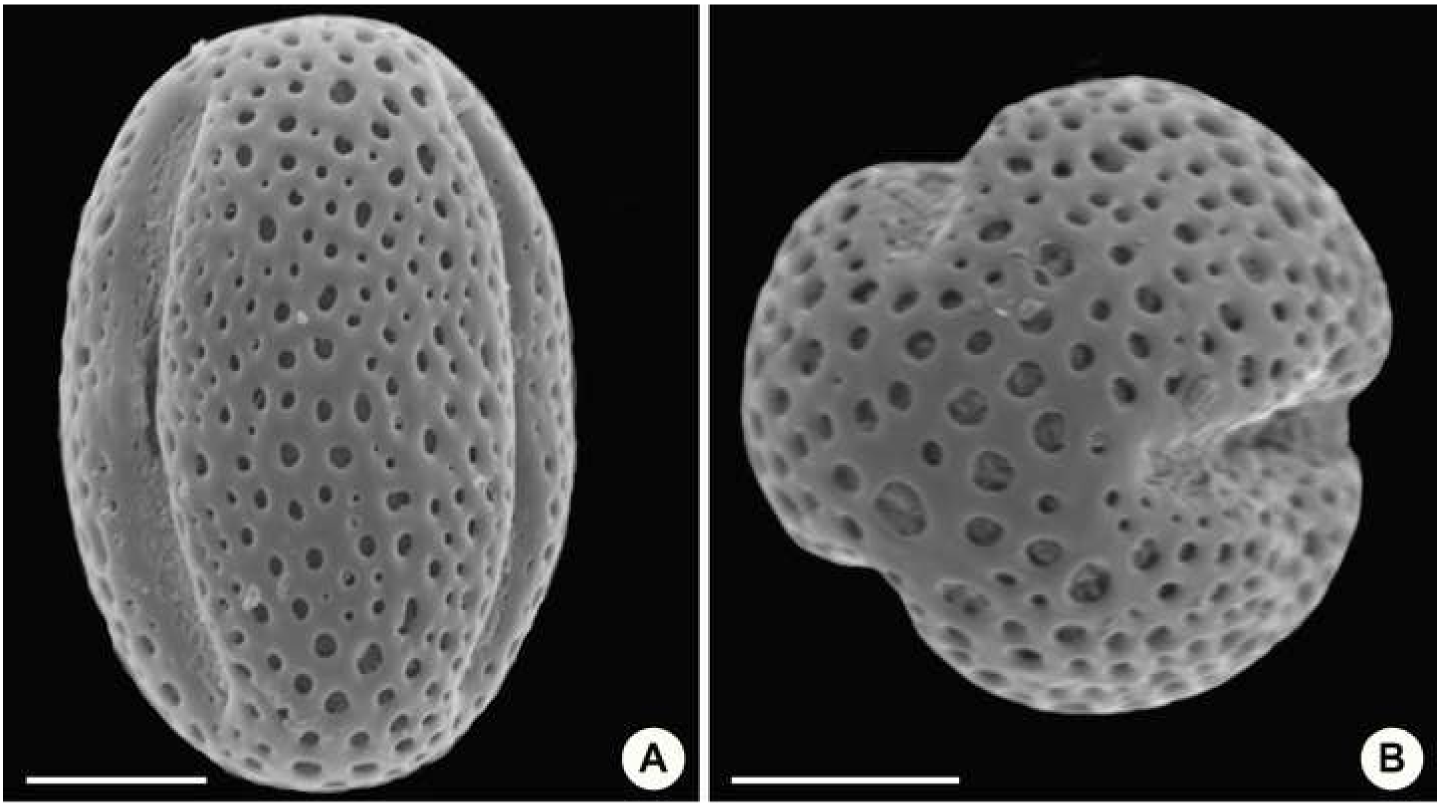

Figure4

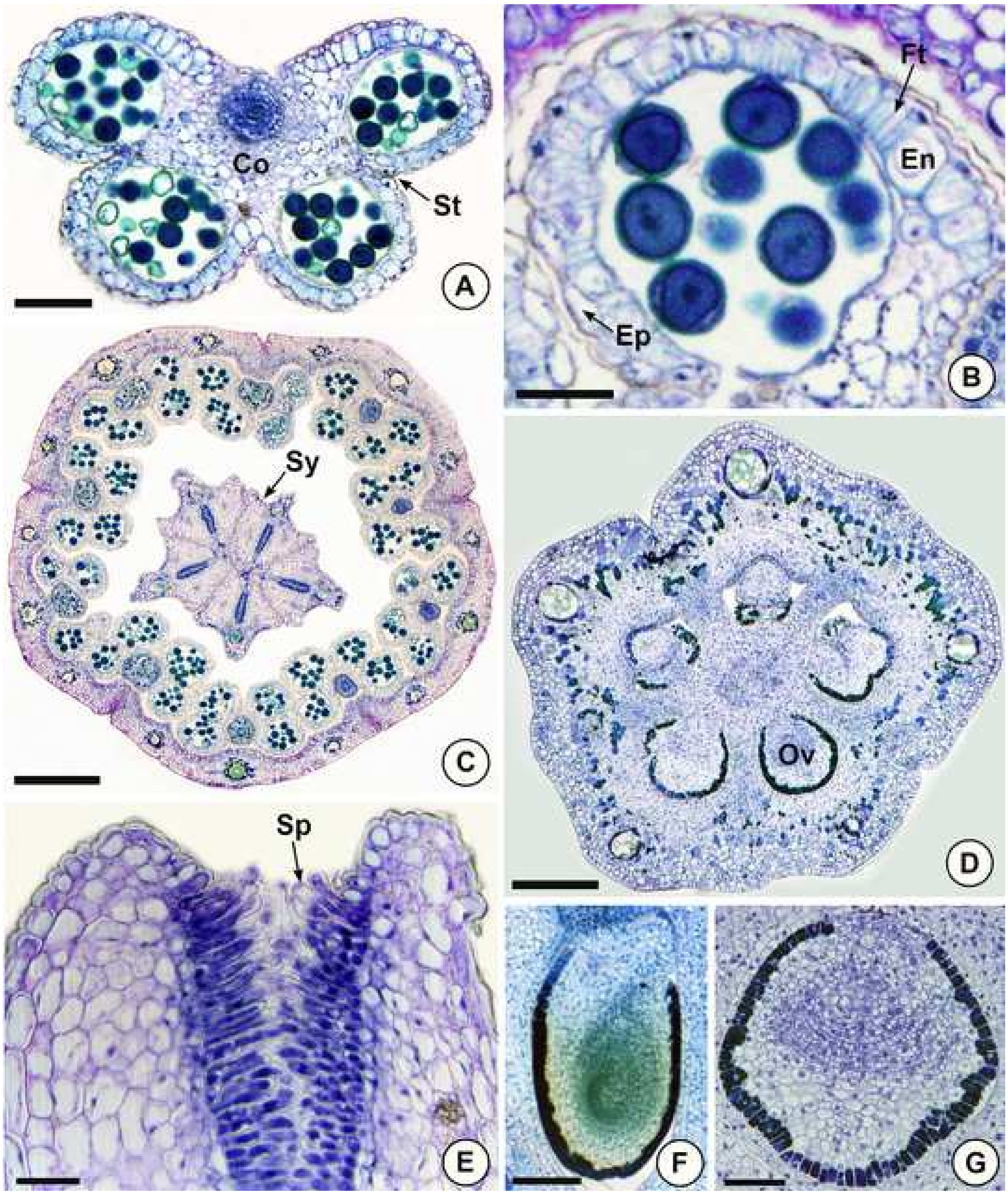

Figure5

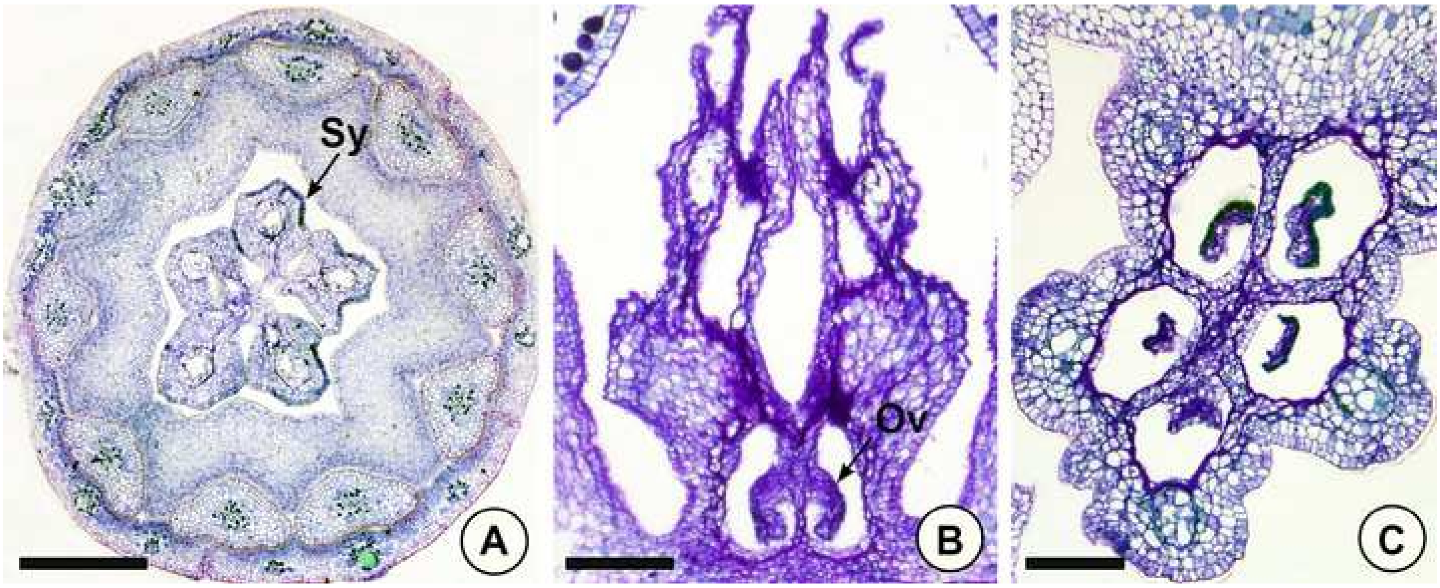

Figure6

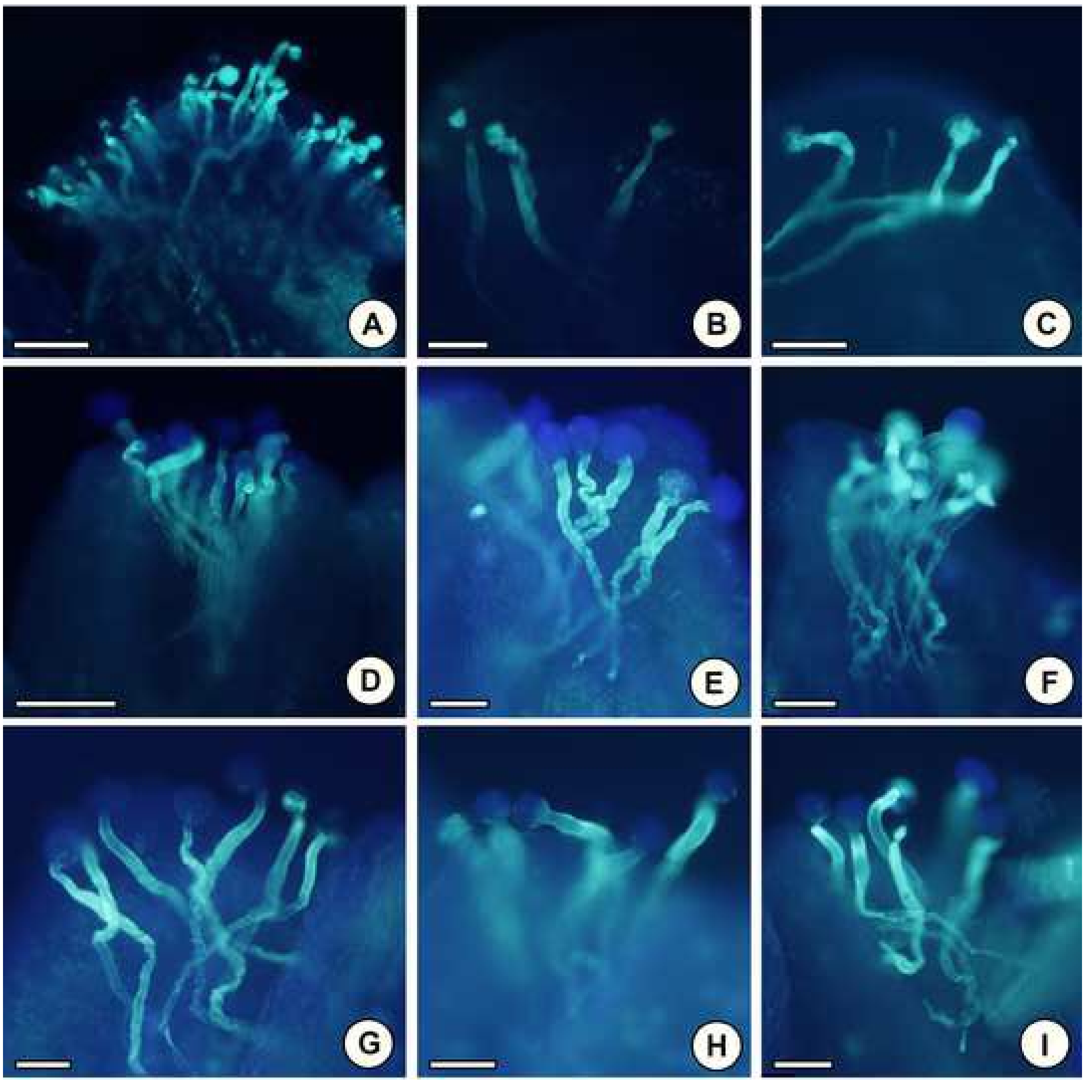

Figure7

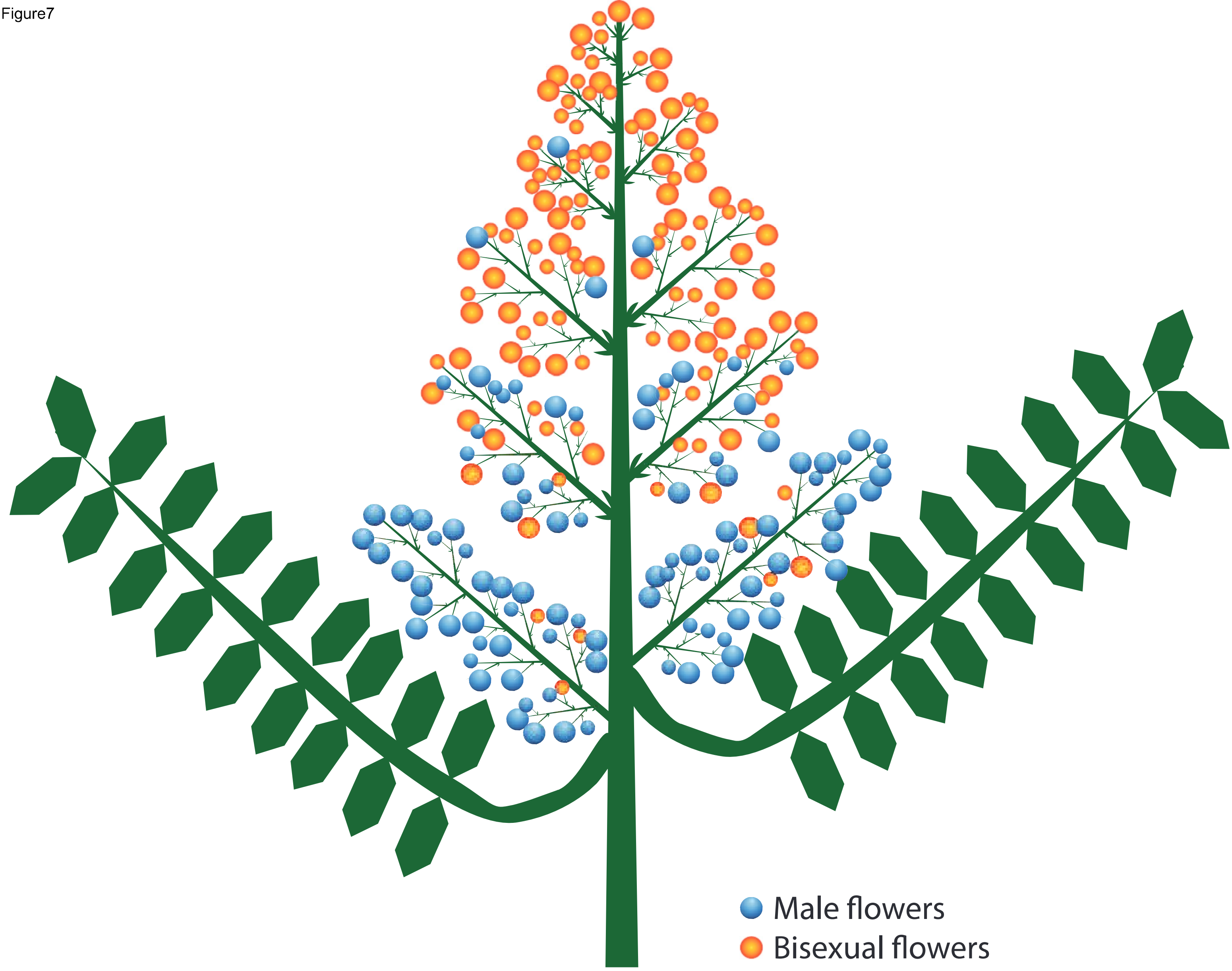

Figure8