



Reproductive system of *Diplopterys pubipetala* (Malpighiaceae) plants from a savanna ecosystem

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ABSTRACT

Diplopterys pubipetala is a Neotropical Malpighiaceae species widely distributed in savanna and tropical semideciduous forests of Brazil. Its flowers are zygomorphic and show glands on calyx, corolla and androecium, which have been related with the attraction, orientation and reward to neotropical oil collecting bees. Nevertheless, previous studies showed differences in the behavior of floral visitors, acting as pollinators or as oil robbers. Additionally, plants from distinct populations may show functional hermaphrodites or male-sterile flowers, the last producing seeds by agamospermy. These data suggest a variation in the reproductive system among populations of *D. pubipetala*. So, the aims of this study were to determine the reproductive system of *D. pubipetala* plants from savanna population in order to confirm the existence of flexibility in the breeding system of this species and to identify the importance of floral visitors for seed set. For this we determined the pollen viability and stigma receptivity using conventional techniques in floral biology research; we also evaluated the morphology and size of pollen grains by anatomical sections of the anthers and the stigma surface by SEM; we performed field experiments to determine the mating system. In the studied area all the individuals of *D. pubipetala* showed flowers with high pollen viability and capability to develop seeds from outcrossing. The stigma receptivity was conditioned to the rupture of stigmatic cuticle, which depended on the bee activity. We also verified the efficiency of *Monoeca* bees in the pollen transportation and deposition on receptive surface of stigma. Our results indicated a predominance of allogamy with a strong dependence on biotic pollen vectors in *D. pubipetala*, although one plant in the study population showed mixed mating system, with seeds produced by agamospermy, self-pollination and cross-pollination. Our data confirm the existence of flexibility in the breeding system of *Diplopterys pubipetala*, with intra and interpopulational variation.

Keywords : agamospermy, bee-pollination, *Monoeca*, outcrossing, self-compatibility.

INTRODUCTION

Malpighiaceae is a pantropical family of approximately 77 genera and 1300 species (Davis & Chase 2004, Davis & Anderson 2010). More than 80% of the species are exclusively Neotropical (Anderson 1979, Davis & Anderson 2010), and the diversity center is in South America (Anderson 2004). In Brazil, approximately 44 genera and 520 species (Mamede *et al.*

2010) are widely distributed throughout different types of vegetation, constituting an important component of “cerrado”, savanna-like vegetation (Ratter *et al.* 1997, Mendonça *et al.* 1998, Anderson 2004, Mamede *et al.* 2010).

Flowers of Neotropical species exhibit morphological uniformity, being basically characterized by zygomorphy, presence of two glands on the abaxial surface of the sepals, five free and clawed petals, ten free stamens and

tricarpellate gynoecium with one ovule per locule and generally three styles (Anderson 1979, Davis & Anderson 2010). These features are related with attraction, orientation and reward of pollinators and probably resulted from the early evolution of an almost obligate and specialized pollination system (Anderson 1979).

Most Malpighiaceae species are predominantly allogamous and highly dependent on their specific pollinators for reproduction (Barros 1992, Teixeira & Machado 2000, Sigrist & Sazima 2004, Costa *et al.* 2006). In fact, female Centridini bees are frequently reported as the main pollinating agents in neotropical Malpighiaceae species (Possobom *et al.* 2015 and references therein) that gather the oil produced in sepal glands (Vogel 1974, Neff & Simpson 1981, Buchmann 1987). The oil is an important resource to the specialized pollinators, which use it to coat and waterproof their nest cells (Neff & Simpson 1981, Simpson & Neff 1981, Buchmann 1987, Vogel 1990) and for larval provisioning (Vogel 1974, Vinson *et al.* 1997, Reis *et al.* 2007).

In addition, even in self-compatible Malpighiaceae species, factors such as dichogamy (protogyny), herkogamy (Sigrist & Sazima 2004, Cappelari *et al.* 2011), and the presence of cuticle recovering the stigmatic surface (Pinheiro *et al.* 2003, Sigrist & Sazima 2004) may avoid the occurrence of spontaneous self-pollination and consequently the autogamy.

Diplopterys pubipetala (A. Juss.) W.R. Anderson & C. Davis is a neotropical Malpighiaceae species that is widely distributed in savanna and tropical semideciduous forests of Brazil (Gates 1982). It is a shrub with vining branches and possesses a variety of secretory structures, including the ones located on the leaves (Possobom *et al.* 2010) and the ones located on calyx, corolla and androecium (Possobom *et al.* 2015), all involved in plant-animal interactions. Sigrist & Sazima (2004) studying the reproductive system of *D. pubipetala* plants growing in a semideciduous forests of Brazil reported pseudo-hermaphroditic, male-sterile flowers with seed set formed only by agamospermy, without legitimate bee visits. However, Possobom *et al.* (2015) studying the same species in savanna vegetation, reported functionally hermaphrodite flowers with dehiscent anthers and legitimate visits mainly by *Monoeca* and *Centris* bees. These new data suggest a variation in the reproductive system among populations of *D. pubipetala*.

In this work we performed manipulative studies aiming to determine the reproductive system of *D. pubipetala* plants from a savanna population in order to confirm the existence of flexibility in the breeding system of this species. Additionally, we investigated the importance of legitimate floral visitors for seed set in these savanna plants of *D. pubipetala*.

MATERIALS & METHODS

Study site and plant species—The study was carried out in a patch of savanna vegetation (called locally as ‘cerrado’) located at Botucatu, São Paulo, Brazil (22°42’38" S and 48°18’35" W). A population of *Diplopterys pubipetala* (n=12 individuals) located at the edge of this savanna remnant was monitored monthly from August 2006 to December 2007 to identify the reproductive phenophases in order to evaluate the reproductive system on the flowering peak.

Voucher samples were deposited in the “Irina Delanova Gemtchujnicov” Herbarium (BOTU) of the Biosciences Institute, University of São Paulo State – UNESP at Botucatu, SP, Brazil (numbers 25306-310).

Analysis of pollen viability and stigma receptivity—Preliminarily, we investigated the pollen viability and stigma receptivity in flowers from all the individuals. The viability of pollen grains was assessed by acetocarmine staining (Dafni *et al.* 2005) (n= 29 flowers, 12 individuals) and by optical microscopy analyses of their morphology and size. Additionally, samples of stamens were fixed in Karnovsky’s solution (Karnovsky 1965) for 24 h, dehydrated in an ethanol series, and embedded in 2-hydroxyethyl-methacrylate resin (Historesin, Leica, Heidelberg, Germany). The sections, that were obtained using a rotatory microtome, were stained with 0.05% toluidine blue, pH 4.3 (O’Brien *et al.* 1964), and mounted in synthetic resin. The images were obtained using a light microscope equipped with a camera. Stigma receptivity was evaluated in stereomicroscope, recording morphological changes, the presence of exudates, colour changes and reaction to hydrogen peroxide (Dafni *et al.* 2005). Additionally, we performed ultrastructural analysis of stigma surface using stigmas from non-visited flowers and from flowers that were exposed to bee visits. The stigma samples were fixed in 2.5% glutaraldehyde (0.1 M

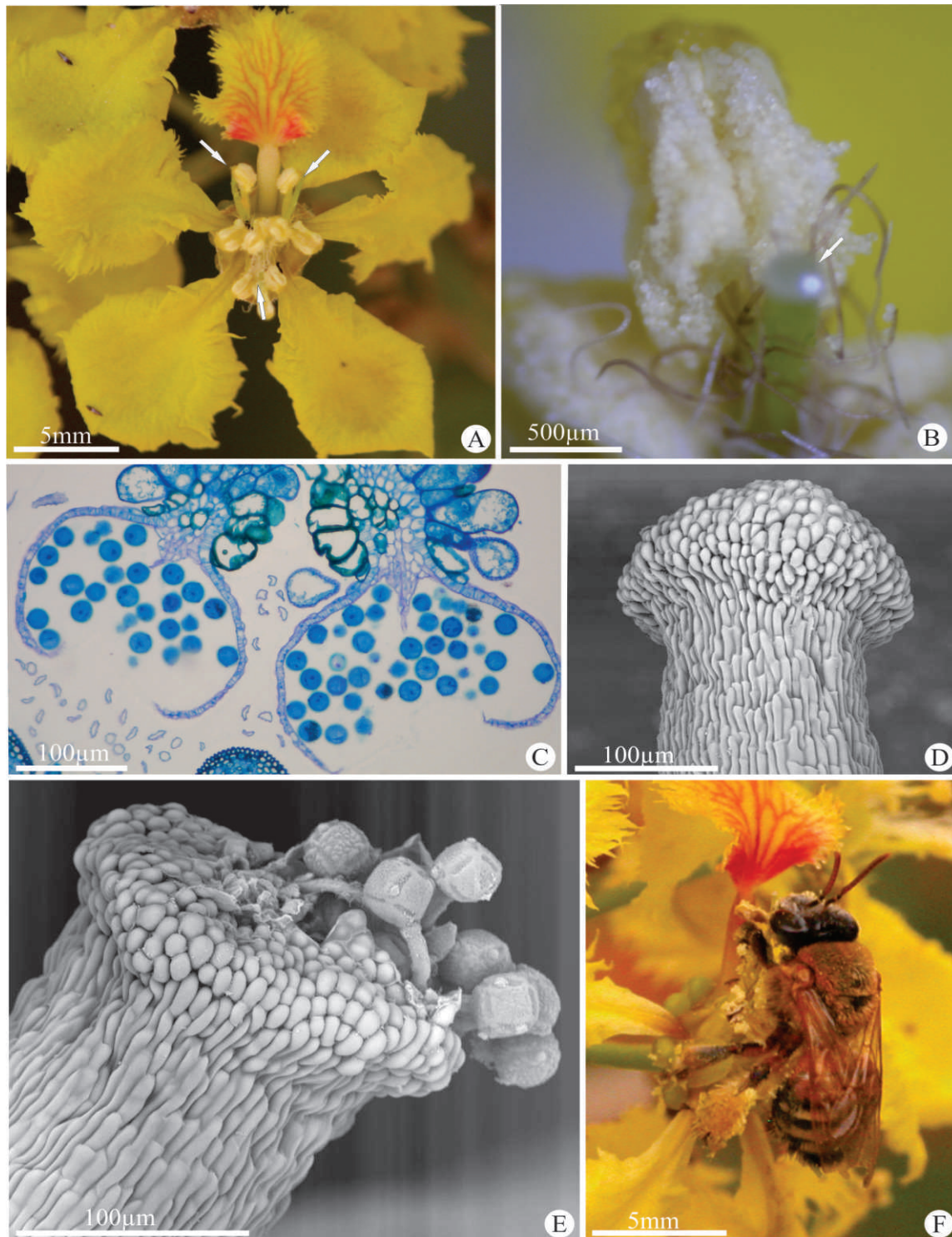


Fig. 1 – Flowers of *Diploteryx pubipetala* (Malpigiaceae). **A.** Flower view showing the five petals, ten stamens and the three stigmas (arrows) positioned in a triangle arrangement. **B.** Stamen with dehiscant anther, fulfilled with an aggregated and white mass of pollen grains and the globose stigma with intact cuticle (arrow). **C.** Cross section of a stamen showing dehiscant anther with viable pollen grains. **D.** Scanning electron microscopy of a globose surface of a non-visited stigma. **E.** Scanning electron microscopy showing stigmatic cuticle ruptured and germinated pollen grains adhered to it. **F.** *Monoeca* sp. visiting a flower legitimately for oil collection.

phosphate buffer, pH 7.3) for 24 hours, post-fixed in 1% osmium tetroxide for 2 hours and dehydrated in an ethyl series. The samples were subjected to critical point drying and were coated with gold using sputter coater. The analysis was made using a FEI Quanta 200 scanning electron microscope.

Reproductive system—The reproductive system of *D. pubipetala* was evaluated from 1020 flowers, bagged since pre-anthesis stage. Considering that each flower can originate up to three samaras (schizocarpic fruit) from the tricarpelar gynoeceum, each potential diaspore was counted as a pollination unit, making a total of 3060 evaluated carpels.

The following treatments were performed on bagged first-day flowers, recently opened. (1) Test for agamospermy (n = 99 flowers, 11 individuals): the flowers were carefully emasculated with appropriate scissors (immediately after flower opening and prior to the dehiscence of the anthers) and kept bagged. (2) Manual self-pollination (n = 47 flowers, 4 individuals): the flowers were pollinated with pollen from the same flower just after anther dehiscence and kept bagged. (3) Spontaneous self-pollination (n = 257 flowers, 8 individuals): the flowers were tagged and kept bagged, without any manipulation. (4) Manual cross-pollination without rupture of stigmatic cuticle (n = 49 flowers, 4 individuals): a mix of pollen from five different individuals (growing at least 20 meters apart) was deposited gently with a small brush, without mechanical pressure, on the stigma surface and the flowers were kept bagged. (5) Manual cross-pollination with artificial rupture of stigmatic cuticle (n=60 flowers, 8 individuals): the flowers were cross-pollinated using the

same mix of pollen (treatment 4) with the application of mechanical pressure using a small brush on the stigmatic surface and were kept bagged. (6) Control with visit: bagged flowers were exposed to visitors for about 40 minutes immediately after opened. We got flowers visited by a *Monoeca* bee. Some of these visited flowers were collected together with non-visited flowers for comparison of stigma surface by scanning electron microscopy (SEM). The remnant flowers (n=12, 1 individual) were kept bagged. (7) Control (n = 496 flowers, 9 individuals): un-bagged flower buds were tagged and monitored for natural pollination. The flowers belonging to treatments 1 to 6 were kept bagged and evaluated periodically for six weeks until flower abscission or fruit development.

RESULTS

We observed buds, flowers and fruits in lower proportions throughout the year in all the *D. pubipetala* plants. The flowering peak occurred at the end of the dry season (August-September) and the mature fruits were dispersed at the beginning of the wet season in November.

Pollen viability and stigma receptivity—The flowers of all the 12 studied individuals of *D. pubipetala* had dehiscent anthers (Fig. 1A-C) and high pollen viability. The average of viable pollen grains per flower was $93.5 \pm 6.8\%$, and only one individual of the study population showed lower average of pollen viability ($83.1 \pm 18\%$). Identification of the stigma's receptive period by conventional field analysis was hindered by the presence of a fine cuticle covering the stigmatic surface, giving it a

Table 1— Experimental pollination and fruit set in *Diplopterys pubipetala* (Malpighiaceae) population from savanna vegetation, at Botucatu municipality, SP, Brazil. (n = 12 plants).

	n	Agamospermy	Self pollination		Cross pollination		Control	Control with visit
			spontaneous	manual	Without cuticle rupture	Without cuticle rupture		
Carpels	3060	297	771	141	147	180	1488	36
Samaras	134	2* (0, 7%)	1 (0, 1%)	7* (4,9%)	3 (2,0%)	39 (21, 7%)	73 (4,9%)	9 (25%)

* These samaras were produced in the individual that presented pollen viability = $83.1 \pm 18\%$ and for which we registered the following fructification rates: agamospermy = 4%, manual self-pollination = 15%, cross pollination = 33%, control = 4%.

dry aspect (Fig. 1B). The thin cuticle covering the stigmatic surface remained intact and exhibited a globose form when the visitors didn't touch it (Fig. 1B, D). When the cuticle was broken, after the application of mechanical pressure, the stigmatic surface revealed a glossy aspect due to the exposure of secretion. Flowers visited by *Monoeca* sp. showed stigma with cuticle ruptured and presence of germinated pollen grains on the surface (Fig. 1E). We verified the adhesion and germination of pollen grains almost immediately after cuticle rupture of *D. pubipetala* stigmas, which were provided by the mechanical friction performed by *Monoeca* sp. during the legitimate visit for oil collection (Fig. 1F).

Reproductive system—In the study population we observed higher fruit set in the cross-pollinated with cuticle rupture and in the bee visited-flowers (Table 1). We registered the formation of fruits by agamospermy in only one individual, which also produced fruits by manual self-pollination, manual cross-pollination and control. The same plant also showed the lowest average of pollen viability.

DISCUSSION

The flowers of all studied plants of *D. pubipetala* were functional hermaphrodites, with female function represented by the seeds produced from natural and manual pollination and male function represented by the pollen grains, with high viability, accessible on the dehiscent anthers. Our findings were different from the previous record of Sigrist & Sazima (2004) that registered male-sterile flowers, with indehiscent anthers and pollen viability of 22% in *D. pubipetala* plants from a semideciduous forest in Southeastern Brazil. Additionally, the importance of the rupture of the stigmatic cuticle for successful pollination in Malpighiaceae species was pointed out by Pinheiro *et al.* (2003) and Sigrist & Sazima (2004). According to these authors, the adhesion, hydration and consequent germination of pollen grains did not occur on stigmas with intact cuticle. In fact, we verified the adhesion and germination of pollen grains almost immediately after cuticle rupture of *D. pubipetala* stigmas by the mechanical friction of *Monoeca* sp.

In a previous study, with the same plant population, Possobom *et al.* (2015) verified that the pollen grains

were naturally exposed at 08:00h, about two hours after the flower opening, when bee visits typically started. In this context, we could predict that in *D. pubipetala* flowers the receptivity of the stigma is conditioned to the cuticle rupture, which generally depends on the pollinators that are visiting flowers when the pollen grains are also available, which favors outcrossing. The importance of the rupture of the stigmatic cuticle for the successful pollination in *D. pubipetala* was clearly indicated by the remarkable increase in seed set after cuticle rupture in cross pollination experiments. So, the mechanical friction exerted by *Monoeca* was effective in influencing *D. pubipetala* reproduction, since it caused the rupture of the stigmatic cuticle, allowing for the adhesion and germination of the pollen grains. Although Vogel (1990) had called into question the role of *Monoeca* species in Malpighiaceae pollination, it is remarkable that flowers visited by a species belonging to this genus produced, proportionally, about five times more fruits than the control, indicating its high efficiency as pollinator of *D. pubipetala*.

In fact, in the study population, our findings showed a predominance of allogamy, since we observed higher fruit set in the cross-pollinated and visited-flowers, which is similar to the mating system reported for the majority of Malpighiaceae species (Barros 1992, Teixeira & Machado 2000, Sigrist & Sazima 2004, Costa *et al.* 2006). In contrast to that verified by Sigrist & Sazima (2004), here we did not find any *D. pubipetala* plant reproducing exclusively by agamospermy. We observed the formation of fruits by agamospermy in only one individual, which also produced fruits by self-pollination and manual cross-pollination thus indicating that it was not an exclusively agamospermous plant, but suggesting a variation in the reproductive system among plants. Although self-compatibility is a relatively frequent trait in Malpighiaceae (Barros 1992, Sigrist & Sazima 2004, Costa *et al.* 2006), factors such as protogyny, herkogamy, and especially the presence of a stigmatic cuticle may hinder the occurrence of spontaneous self-pollination (Sigrist & Sazima 2004) and enhance the importance of biotic vectors in order to allow the adequate transfer of pollen from anthers to a receptive stigma surface.

Our data indicate the occurrence of both interpopulational and intrapopulation variation in the

functionality of *D. pubipetala* flowers, suggesting a mixed reproduction system in this plant species. According to Rowlands (1964), self-incompatibility is rarely complete in nature. Although many authors refer to plant species simply as self-compatible or self-incompatible, their reproduction systems are more complex and different levels of self-compatibility may exist among the individuals of a single population (Lipow & Wyatt 2000). Similar to what Barcaccia *et al.* (2006) observed for facultative apomictic ecotypes of *Hypericum perforatum* (Clusiaceae), the reproductive strategies in *D. pubipetala* seem to be dynamic and versatile. Considering the occurrence of *D. pubipetala* in disturbed areas such as edges of forest fragments and roadsides, this variation may give the species a greater potential for occupying these environments, facilitating the dissemination of locally adapted genotypes through agamospermy, and on the other hand, allowing for genetic recombination through sexual reproduction.

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