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FLORAL BIOLOGY AND POLLINATION OF *MANIHOT* MILL. (EUPHORBIACEAE), WITH EMPHASIS ON TWO SPECIES OF THE CERRADO

BIOLOGIA FLORAL E POLINIZAÇÃO DE *MANIHOT* MILL. (EUPHORBIACEAE), COM ÊNFASE EM DUAS ESPÉCIES DO CERRADO

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Dissertação apresentada ao Instituto de Ciências Biológicas da Universidade de Brasília (UnB) como parte dos requisitos exigidos para obtenção do Título de Mestra em Botânica

Orientador: Prof. Dr. Hélder Nagai Consolaro

Coorientador: Prof. Dr. Antonio José Camillo de Aguiar

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DEDICATÓRIA

Ao Cerrado, por sua imensa diversidade de formas, que abriga tamanha fonte de riqueza e inspiração.

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RESUMO

As espécies do gênero *Manihot*, das quais *M. esculenta* apresenta importância alimentícia, são pouco conhecidas sobre sua biologia reprodutiva. Nesta dissertação investigamos caracteres florais, reprodutivos e interações planta-polinizador em *Manihot*, focando em espécies do Cerrado, bioma com área prioritária para conservação do gênero. No primeiro capítulo, comparamos espécies de distribuição restrita no Cerrado a espécies mais amplamente distribuídas. Revelamos a ocorrência de osmóforos, glândulas que emitem odores, nas flores de todas as 16 espécies analisadas. As estruturas das glândulas florais mantiveram-se conservadas e aplicando testes histoquímicos em três espécies, identificamos compostos que potencialmente contribuem na proteção das flores e atração de polinizadores (terpenos). As espécies endêmicas do Cerrado se diferenciaram no design da flor feminina e morfologia dos osmóforos, indicando maior diversificação nesse bioma. No segundo capítulo, demonstramos em duas espécies de *Manihot* do Cerrado, sistemas reprodutivos que dependem de polinizadores para a formação de sementes. Variados grupos funcionais de insetos atuaram como polinizadores, porém as espécies podem manifestar características florais, como baixa concentração do néctar, que resultam na restrição de determinados visitantes. As duas espécies diferem nos principais polinizadores (abelhas *versus* formigas e moscas) e, independente do sistema de polinização generalista, evidenciamos filtros florais. Ademais, a monoicia limita possibilidades de visitantes pouco frequentes polinizarem pelo baixo número de flores femininas. Concluindo, apesar de possuir características florais conservadas, as espécies de *Manihot* podem diferir em sua biologia da polinização, tendendo a depender de grupos de insetos mais abundantes para seu sucesso reprodutivo.

Palavras-chave biologia reprodutiva da mandioca, endemismo, flores unissexuais, interações planta-inseto, metabólitos secundários, parentes silvestres de culturas

ABSTRACT

The species of the genus *Manihot*, whose *M. esculenta* is of nutritional importance, are poorly known about their reproductive biology. In this dissertation we investigated floral and reproductive characters and plant-pollination interactions in *Manihot*, focusing on species of the Cerrado, a biome with priority area for conservation of the genus. In the first chapter, we compared species with restricted distribution in the Cerrado to more widely distributed species. We revealed the occurrence of osmophores, scent glands, in the flowers of all 16 species analyzed. The structures of the floral glands were conserved and applying histochemical tests in three species, we identified compounds that potentially contribute to the protection of the flowers and attraction of pollinators (terpenes). The endemic species of the Cerrado differed in the design of the female flower and morphology of osmophores, indicating greater diversification in this biome. In the second chapter, we demonstrated in two Cerrado *Manihot* species, reproductive systems that depend on pollinators for seed formation. Various functional groups of insects acted as pollinators, but the species can manifest floral characteristics, such as low concentration of nectar, resulting in the restriction of certain visitors. The two species differ in the main pollinators (bees versus ants and flies) and, regardless of the generalist pollination system, we evidenced floral filters. Additionally, monoecy limits the possibilities of infrequent visitors to pollinate due to the low number of female flowers. In conclusion, despite having conservative floral traits, *Manihot* species can differ in their pollination biology, tending to depend on more abundant groups of insects for reproductive success.

Keywords crop wild relatives, endemism, plant-insect interactions, reproductive biology of cassava, secondary metabolites, unisexual flowers

SUMÁRIO

GENERAL INTRODUCTION

Manihot is a neotropical genus with recent diversification in South America, reaching the highest diversity and endemism in Brazilian Cerrado (Rogers and Appan 1973, Chacón *et al*. 2008, Duputié *et al*. 2011). The genus is recognized worldwide due to cassava (*M. esculenta*), whose tuberous root is an important food source of carbohydrate for over 700 million people (http:// faostat.fao.org). Cassava has a great potential for improvement by gene pool from the wild relatives of the species (Nassar and Hashimoto 2006, Nassar *et al*. 2008, 2012, Castañeda-Álvarez *et al*. 2016). However, many *Manihot* species are endangered (IUCN 1997). Furthermore, from Cerrado a high proportion (41%) of endemic species are threatened, mainly due to habitat loss (Simon *et al.* 2018).

Manihot presents unisexual flowers (Rogers and Appan 1973), which is uncommon amongst flowering plants (Tanurdzic and Banks 2004). The genus features well-conserved floral structure, with flowers in a single whorl formed by five tepals and have on the bottom of the flower a nectariferous gland (Rogers and Appan 1973). The flowers are insect pollinated, mainly by bees and wasps (Rogers and Appan 1973, Nassar and Carvalho 1990). *Manihot* flowers are often dish to bowl shape that is thought to be generalist pollinated (Olesen *et al.* 2007). Although, studies in natural conditions are lacking for the genus and interpretations about pollination systems based solely on floral morphology can be misleading (Lindsey and Bell 1985, Ollerton *et al*. 2007). Consequently, here, we should look at field conditions to understand plant-pollinator interactions.

According to Rogers and Appan (1973), the sexual system of the genus is predominantly monoecious (individuals bearing both unisexual flowers), with a minority of dioecious species (unisexual individuals). This is consistent with what is known for the Euphorbiaceae, whereby the monoecious system is predominant (Webster 1994, Bullock 1985, Machado *et al.* 2006, Chen and Li

2008). Moreover, monoecy can be associated with protogyny (Bertin and Newman 1993), indeed *Manihot* species are protogynous at inflorescence level (Halsey *et al*. 2008, Perera *et al*. 2013): female flowers function prior to male flowers, therefore the sexes are temporally separated (dichogamy). The dichogamy evolves in the presence of sex interference and inbreeds depression, though the match between the time of pollen dispersal and available ovules commonly results in asymmetric dichogamy (Sargent *et al*. 2006). Despite that, the protogyny evolution act minimizing the sex interference, increases the crossing rate and potentially avoid self-fertilization (Bertin 1993, Barrett 2003, Narbona *et al*. 2011, Pang *et al*. 2013). These premises can be applied to *Manihot* species, in which the breeding system is out-crosser (McKey *et al*. 2005), but sometimes male and female flowers are open at the same time and geitonogamy becomes possible (da Silva *et al*. 2003, Lai *et al*. 2014).

In flowers with separated sexes (space or time), different floral traits might evolve (Hemborg and Bond 2005, Barrett and Hough 2013). Flower size dimorphism tends to be predominant in taxa bearing unisexual flowers, whereby 85% revealed sexual dimorphism in size; in addition, the relative size was suggested to be influenced by the climate zone: in the temperate zone the male flower is often larger, and in tropics zones both sexes display equally odds to be larger (Delph *et al.* 1996). Flower size dimorphism is also conspicuous to endemic taxa (Humeau *et al.* 2003). In *Manihot*, Byrne (1984) reported that *M. esculenta* female flowers are twice the size of male flowers. Although in Cerrado species the male flowers bigger than females seems prevalent (Silva and Sodré 2014, Mendoza *et al*. 2015, 2016). Additionally, *Manihot* present sexually dimorphic perianth, with the female flowers dish-shaped and on the other way, the male flowers are generally bell-shaped. Plants bearing unisexual flowers allow comprehension of selection pressures on each sexual component (Costich and Meagher 2001, Rocheta *et al*. 2014). However, shape dimorphism still appears a poorly explored subject (Miller and Venable 2003). The evolutionary pressures resulting in the dimorphic shape of *Manihot* unisexual flowers could represent a matter of interest.

Considering the unexplored fields in the reproductive biology of *Manihot* and its main center of endemism in Cerrado, we proposed different perspectives in this study. In the first chapter, the focus is on the *Manihot* flowers, in which we investigated two floral glands known for mediate interactions with pollinators – osmophores and nectaries – providing new information for the genus. Besides, we reviewed floral features and support the idea that Cerrado endemic species manifested diversified traits among the genus. The second chapter presents the pollination and breeding systems of two wild *Manihot* species of Cerrado. In addition, we evaluated floral biology in male and female flowers giving insight into pollinator filters functioning in a monoecious plant.

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CHAPTER 1

Optimizing gland functions: defense and attraction evidence in conservative floral structures of *Manihot* species (Euphorbiaceae)

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ABSTRACT

Floral traits evolution undergoes selective pressures by antagonists and mutualists, i.e. secretory structures and glands function for defense and pollinators attraction. We explored floral features and glands of *Manihot*, to give insights into the genus floral evolution. Furthermore, we evaluated if narrowly distributed species of the main diversity center of the genus – Cerrado – present differential floral traits. We studied 16 species, eight narrowly distributed in Cerrado and eight more widely distributed or from a different biome. We reviewed in literature the species floral traits, examined its flowers and performed red neutral test to detect secretory activity. In three species, we analyzed the structure and histochemistry of the glands. Our data supported two floral glands in *Manihot*, nectary, and osmophores (scent gland), providing the second register of osmophores in Euphorbiaceae. Narrow endemic species of Cerrado had differential floral features in female flower morphology and size and more often had deciduous bracts. Species endemic to Cerrado also appeared with more conspicuous osmophores. Our findings indicate that the Cerrado species evolved differential floral traits in *Manihot*. Although species presented individual variation in floral traits, a very conservative state for both floral glands evinced. Both glands had secretory structures and compounds related to defense and pollinator attraction. Due to osmophore pattern in tepals and its main chemicals, it seems that this gland evolved first to function in floral protection. In summary, a double role of floral glands in attraction and defense should be common, especially in osmophores, thereby optimizing plant reproductive fitness and defense system.

Keywords alkaloids, cassava, endemism, monochlamydeous, monoecy, scanning electron microscopy, terpenes, unisexual flowers

INTRODUCTION

Floral glands have multiple independent origins and its secretion is known to mediate complex interactions of plant-pollinators since the early evolution of angiosperms (Pacini *et al.* 2003, Bernardello 2007, Nepi *et al.* 2009, Erbar 2014, Poinar and Poinar 2018). Nectar is probably the most common floral reward and it can be an innovation that increases reproductive success among animal pollinated taxa (Simpson and Neff, 1981, Neiland and Wilcock 1998, Tong *et al.* 2018). In pollination, besides rewarding glands, there are also scent glands (or osmophores) that act in plant-pollinator communication by floral volatile emission (Vogel 1990, Piechulla and Pott 2003, Raguso 2008). A variety of tissues can emit floral scents, in specialized areas or in a diffuse way, occurring in different organs, such as petals, sepals, anther connective, and receptacle (Vogel 1962, Sazima *et al.* 1993, Vogel and Hadacek 2004, Bergougnoux *et al.* 2007, Kowalkowska *et al.* 2014, Possobom *et al.* 2015, Wiśniewska *et al.* 2018). The importance of scent emission in floral evolution is yet to be highlighted. In fact, odor signals in floral recognition, mainly for insect pollinators, can constitute an accurate and honest signal of rewards, even greater than flower size and color (Knudsen *et al.* 2001, Wright and Schiestl 2009, Parachnowitsch *et al.* 2012). Moreover, higher emission of floral scents is related to a higher reproductive fitness of the plants (Majetic *et al.* 2009).

In addition to floral glands acting in pollinator rewarding and attraction, defense function is found for both scent glands and nectaries (Domínguez *et al.* 1989, Thornburg *et al.* 2003, Narbona and Dirzo 2010, Marques *et al.* 2015, Płachno *et al.* 2017). Compounds of floral scents and nectar can simultaneously attract pollinators and defend against antagonists (Junker and Bluthgen 2010, Junker *et al.* 2011, Huang *et al.* 2012). The dual issues of plant reproduction and defense have been shown recurrent in floral traits (Strauss 1997, Pichersky and Gershenzon 2002, Irwin *et al.* 2004, Sun *et al.* 2008, Kessler *et al.* 2008, Kessler and Halitschke 2009, Johnson *et al.* 2015, Sletvold *et al.* 2015, Veiga *et al.* 2015, Lucas-Barbosa 2016), despite that works about only one gland function predominate in the literature (e.g. Fahn 2000, Machado *et al.* 2015, Demarco 2017, Płachno *et al.* 2018).

The family Euphorbiaceae for example, possesses both secretory structures related to defense such as, laticifers, colleters, idioblasts, trichomes, and structures for pollinator attraction such as nectaries, resin reward, and osmophores (Armbruster 1984, Freitas *et al* 2001, Machado *et al.* 2015, Vitarelli *et al.* 2015, Gagliardi *et al.* 2016). The family is noteworthy by the diversity of secondary compounds (Solereder 1908, Metcalfe and Chalk 1950, Thomas 1991, Seigle 1994). Another remarkable feature is the modified inflorescences (cyathium) that are highly specialized (Prenner and Rudall 2007, Thakur and Patil 2011), even though restricted to the tribe Euphorbieae (Steinmann and Porter 2002). Nevertheless, in the floral evolution of Euphorbiaceae, reductions in number and types of whorls are frequent (Venkata-Rao and Ramalakshmi 1968, Webster 1994, De-Paula *et al.* 2011). Simultaneously to floral morphology evolution, the glands also diversified within euphorbiaceous lineages (Armbruster 1985, Cacho *et al.* 2010).

Manihot is a monophyletic genus of Euphorbiaceae from Neotropical region that probably originates in Mesoamerica, and recently (ca 3.5 Ma) radiated in South America (Chacón *et al*. 2008, Duputié *et al.* 2011). *Manihot esculenta*, the cassava, is worldwide recognized as an important crop for human food supplier (Hershey 2010). It is known that the mating system of *M. esculenta* is predominantly outcrossing and flowers are insect pollinated (Rogers and Appan 1973, Nassar and Carvalho 1990, da-Silva *et al.* 2003, Lai *et al.* 2014). The related wild species are a potential resource for the genetic improvement of commercial species (Nassar *et al.* 2012, An *et al*. 2016). The main diversity and endemism of *Manihot* are in the Cerrado of central Brazil, with 75 species (Rogers and Appan 1973, Duputié *et al.* 2011, Simon *et al.* 2018). The Cerrado is the highest priority area for conservation of cassava wild relatives (Castañeda-Álvarez *et al.* 2016) and many of its endemic species are currently threatened (Simon *et al.* 2018). It was revealed that many Cerrado lineages are derived and recent, which diversified in situ and adapted to frequent fires (Simon *et al.*

2009). In fact, the *Manihot* appeared to rapidly evolve in this biome (Duputié *et al.* 2011). A study with narrow endemic species supports differential reproductive traits comparing to its wide-spread congeners (Lavergne *et al*. 2004). Therefore, we could expect differential floral traits in endemic *Manihot* species of Cerrado.

Despite that, the genus presents a well-conservative floral structure, with unisexual flowers reduced in a single whorl formed by five tepals (Rogers and Appan 1973, Webster 1994, Carmo Júnior *et al*. 2013). Certainly, some traits appear constant in *Manihot* flowers as the nectary named basal or floral disk (Venkata-Rao and Ramalakshmi 1968, Rogers and Appan 1973, Perera *et al.* 2013); though the nature of secretion or gland structure was not reported. In addition, we recognized a modified region in tepal margins, yellow glandlike, which was not studied so far. Overall, floral features of the genus remain untapped. Therefore, we aimed to explore the following questions in *Manihot* floral evolution: (1) how conservative are the floral traits of *Manihot* in South America, more specifically, in Cerrado? Do the endemic species with a narrow distribution in Cerrado present different floral traits? (2) Which are the floral glands and secretory structures in *Manihot* and how conservative are they? What are the possible floral gland functions?

MATERIALS AND METHODS

Studied species

We studied 16 species of *Manihot* belonging to four of the seven subclades of *Manihot* phylogeny, mainly from dry climates (Duputié 2011). Most investigated taxa are endemic to Cerrado, as following: *M. congesta* M. Mend. & T.B. Cavalc., *M. debilis* M. Mend. & T.B. Cavalc., *M. fruticulosa* (Pax) Rogers & Appan, *M. minima* M. Mend. & T.B. Cavalc., *M. pinatiloba* M. Mend. & T.B. Cavalc., *M. porphyrantha* M. Mend. & T.B. Cavalc., *M. purpurea* M. Mend. & T.B.

Cavalc., *M. robusta* M. Mend. & T.B. Cavalc. and *M. sagittata* M. Mend. & M. Martins. The material of these taxa was obtained from the living collection of the Brazilian Agricultural Research Corporation (Embrapa)/Genetic Resources and Biotechnology (Cenargen) collected originally from in-situ populations under collection numbers of M. Mendoza (see **Table 1** for voucher details). The *M. esculenta* was included in our samplings and assessed from a cultivar from Embrapa Cerrados. Additionally, *M. glaziovii* Müell. Arg. and *M. pseudoglaziovii* Pax & K. Hoffm. from Caatinga biome were obtained from the living collection of the Estação Biológica da Universidade de Brasília. Other species were collected in their natural habitat in Cerrado: *M. gracilis* Pohl, *M. oligantha* Pax, *M. tripartita* (Spreng.) Müll.Arg. and *M. violacea* Pohl.

Floral traits in Manihot and species distribution range

We reviewed from the literature the floral traits and biome for the analyzed species (Rogers and Appan 1973, Byrne 1984, Mendoza *et al.* 2015, 2016, 2018, Mendoza and Martins 2018). Data of floral anthesis, nectar traits and pollinators are largely unknown for the wild species. Therefore, the assessed information was according to its availability for most species, which consisted of bracts features, male and female flower shape, length size (mean of the maximum and minimum measurements) and flower color.

We used the distribution range provided by the recent study of Simon *et al.* (2018), in which we established an endemism criterion based on the extent of occurrence (EOO) of the species. Defining narrow endemism can be very difficult and several factors should be considered (Kruckeberg and Rabinowitz 1985), thus it might explain why there is not an absolute definition (Médail and Baumel 2018). Here, we considered that species with EOO smaller than 170 km² as narrow distributed (see Pisanu *et al.* 2009).

Gland morphology and secretory activity

To assess floral glands, its morphology, the presence of secretion and possible differences between sexes, we collected fresh flowers from the studied species and examined under a stereoscopic microscope (Leica M125). Plant material was collected according to its availability, but often we were limited by the low number of available flowers in anthesis. Despite that at least six intact flowers from each species were submerged in neutral red to detect secretory activity (Vogel 1990) and photographed before and after the test (AmScope FMA050).

Gland structure in scanning electron microscopy

For micromorphology analyses, we carried out scanning electron microscopy (SEM) in the floral location with secretory activity. We selected three species, *M. esculenta*, *M. violacea*, and *M. oligantha,* based on the availability of flowers. Open fresh flowers were fixed in formaldehyde-glutaraldehyde 2.5% for 24h (Karnovsky 1965) and conditioned in phosphate buffer 0.1 M, pH 7.3. The samples were post-fixed with osmium tetroxide 1% for 30 min, dehydrated in acetone series, dried at the critical point using liquid CO_2 (Balzers, CPD 030), mounted on stubs, coated with a golden layer (Balzers, SCD 050) and analyzed in SEM (Jeol JSM-7000F).

Gland structure in optical microscopy

For inner secretory structure analysis, we collected male and female flowers from the same species submitted to SEM. We sampled recent open flowers and fixed in neutral buffered formalin during 48h (Lillie 1965), then embedded in histological paraffin (Johansen 1940). The longitudinal and transversal sections 8-10 µm thick were obtained in a rotary microtome and stained with safranin and toluidine blue (Johansen 1940). The slides were mounted in synthetic resin (Paiva *et al.* 2006). Additionally, we applied a different protocol for minute regions on tepals with secretory activity, for the species *M. esculenta* and *M. violacea*. Initially, the procedure of fixation and post-fixation followed as described for SEM. Afterward, we added uranyl acetate for contrast and embedded the samplings in epoxy resin (Araldite). Semi-thin sections with 70-80 nm thick were obtained using ultra-microtome and stained with toluidine blue 1%.

Histochemical analysis

For histochemical analyses of the floral glands, we prepared the samples following the protocol described above for paraffin (Johansen 1940), with exception of the reagents for alkaloids and terpenes detection that we used fresh samples, sectioned by hand-cut. We employed histochemical tests for the main compounds classes detection: 1) lugol (iodine and potassium iodide) for starch (Johansen 1940); 2) periodic acid/Schiff reagent for polysaccharides (McManus 1948); 3) Comassie R250 for protein (Fisher 1968); 4) ruthenium red for acid mucilage (Gregory and Baas 1989); 5) Sudan IV and Sudan Black B for total lipids (Pearse 1985); 6) ferric chloride III for phenolic compounds (Johansen 1940); 7) Wagner's reagent for alkaloids (Furr and Mahlberg 1981); 8) NADI for terpenoids (David and Carde 1964). The control and slide mounting followed the procedure of the respective test. The sections were analyzed and the results were registered in a photomicroscope Olympus, U-TV0.5xC-3 with the capture system Olympus SC30.

RESULTS

Floral traits in Manihot species and distribution range

We found eight species restricted to the Cerrado and eight more widely distributed or present in other biomes. Floral bracts had diversity in its morphology, being discreet when setaceous and more conspicuous when ovate, though many were deciduous (**Table 1, Figure 1**). The deciduousness appeared more in the narrow endemics of Cerrado (six out of eight) and less in the wider distributed species (two out of eight). Flowers, in general, were campanulate and all male flowers were campanulate to tubular-campanulate with united tepals (gamotepalous). The female flowers had free

Table 1 *Manihot* sampled species. Taxa with restricted distribution are indicated as narrow

Species	Distribution	Bracts	Floral shape (♀ x ♂)	Floral length (♀ x ♂, mm)	Staining by neutral red	Voucher
M. esculenta	cultivated	setaceous (d)	campanulate (p) x campanulate (g)	13.0 x 5.0	A, M, P	H. Farinasso A12, UB
M. tripartita	Amazon, Cerrado,	ovate	campanulate (p)x campanulate (g)	7.0 x 9.0	A, M, P	H. Farinasso A8-9, UB
M. pseudoglaziovii	Caatinga	setaceous	campanulate (p) x campanulate (g)	12.0	A, M	H. Farinasso A6, UB
M. glaziovii	Caatinga	setaceous	campanulate (p) x campanulate (g)	15.0	A, M, P	H. Farinasso A5, UB
M. fruticulosa	Cerrado	setaceous	campanulate (p) x campanulate (g)	8.0 x 8.0	A, M, C	M. Mendoza et al. 4801
M. gracilis	Cerrado	lanceolate	campanulate (p) x short-campanulate (g)	7.5 x 8.0	A, M, P	H. Farinasso A7, A10-11, UB
M. violacea	Cerrado	ovate-lanceolate	campanulate (p) x campanulate (g)	10.8 x 13.2	A, M, P	H. Farinasso A3-4, UB
M. congesta	Cerrado	lanceolate (d)	campanulate (p) x tubular-campanulate (g)	7.0 x 9.5	A, M, C	M. Mendoza et al. 5294, CEN
M. debilis	Cerrado, narrow	setaceous (d)	tubular- campanulate (g) x tubular- campanulate (g)	9.0 x 12.0	A, M, P	M. Mendoza et al. 4396, CEN
M. minima	Cerrado, narrow	linear-lanceolate	campanulate (g) x campanulate (g)	7.0 x 7.5	A, M, P	M. Mendoza et al. 4405, CEN
M. oligantha	Cerrado, narrow	setaceous	campanulate (p) x campanulate (g)	6.8 x 8.6	A, M	H. Farinasso A1-2, UB
M. pinatiloba	Cerrado, narrow	linear-lanceolate (ned)	campanulate (p) x campanulate (g)	6.0 x 9.0	A, M, P	M. Mendoza et al. 4341, CEN
M. porphyrantha	Cerrado, narrow	linear-lanceolate (d)	campanulate (g) x campanulate (g)	7.5 x 14.5	A, M, C	M. Mendoza et al. 5020, CEN
M. purpurea	Cerrado, narrow	ovate-lanceolate (ned)	campanulate (g) x tubular-campanulate (g)	8.5 x 10.0	A, M, P	M. Mendoza et al. 4386, CEN
M. robusta	Cerrado, narrow	ovate-lanceolate (ned)	short-campanulate (g) x short-campanulate (g)	8.0 x 10.5	A, M, C, P	M. Mendoza et al. 4354, CEN; UB
M. sagittata	Cerrado, narrow	subelliptic (d)	tubular- campanulate (g) x tubular- campanulate (g)	9.5 x 12.5	A, M, C, P	M. Mendoza et al. 5101, CEN

Abbreviations: bracts deciduousness (d, deciduous; ned, not early deciduous), female (♀) and male (♂) flowers, tepal union (g, gamotepal; p, polytepal), tepal region stained with neutral red (A, apex; M, margins; C, central tepal; P, patchy pattern).

tepals (polytepalous), but six species narrowly distributed in Cerrado featured female flowers with united tepals, similar to male flowers (e.g. *M. porphyrantha*). We notice that the campanulate shape was variable among species, from short-campanulate, subglobose to tubulose (**Fig. 1e, f, h**). The flowers measured between 6-15 mm, characterized as small size and male flowers tended to be relatively larger than female flowers (except in *M. esculenta*). Flowers had green, white, yellow, purple coloration, and sometimes combined colors (result showed for tepals in **Fig. 2**). However, individuals can differ in flower color (green or yellow, even purple to green) and purplish tinge in the same species. Likewise, bracts can vary among individuals of a species (**Fig. 1a, i**).

All *Manihot* species had osmophores in their tepals, with secretory epidermis identified by neutral red staining (**Fig. 3**). The positive staining with neutral red evidenced two main osmophore patterns on the ventral tepals: 1) diffused secretory epidermis on the tepal surface; 2) papillae on tepal distal margin and apex. The osmophore had rough areas formed by papillae, shining papillae-like trichomes and also trichomes.

The osmophore localization among species was very conservative within flower perianth: evident in the margin and apex of two tepals, discreet and more restricted to the apex in two others, and half of the structure is visible on the last tepal (**Fig. 4**). Furthermore, certain species only had discreet osmophores (*M. gracilis*, *M. tripartita*, **Fig. 3a, m**), even though they followed the mentioned pattern. These species and also *Manihot glaziovii* from Caatinga had specific colors within the pattern, e.g. white, green and half white half green (**Fig. 2m**). The surfaces most enlarged were from osmophores of Cerrado species (**Fig. 2b, f-l**). Trichomes were a common feature and did not appear only in *M. pinatiloba*. In general, trichomes were not constant in the same tepal in size

Figure 1 Inflorescences and flowers in *Manihot* species to illustrated the floral diversity of the genus, all the species occur in Cerrado. **a** Conspicuous bracts of *M. tripartita*. **b** Showy bracts of *M. violacea*. **c** Setaceous bracts of *M. veadeirensis*, with *Apis mellifera* visiting the male flower. **d** Discreet bracts (arrow) of *M. oligantha*. **e** Wide openned male flowers of *M. gracilis*. **f** *M. nana* campanulate male flower **g** Tubular-campanulate male flowers of *M. incisa*. **h** Tubular male flower of *M. tenella*. **i** Female flowers of *M. tripartita*, note that the bracts do not appear as in (a). **j** Wide open female flower of *M. robusta*. **k** Campanulate female flower of *M. incisa* with pink stigma. **l** Intense purple and campanulate female flower of *M. porphyrantha*.

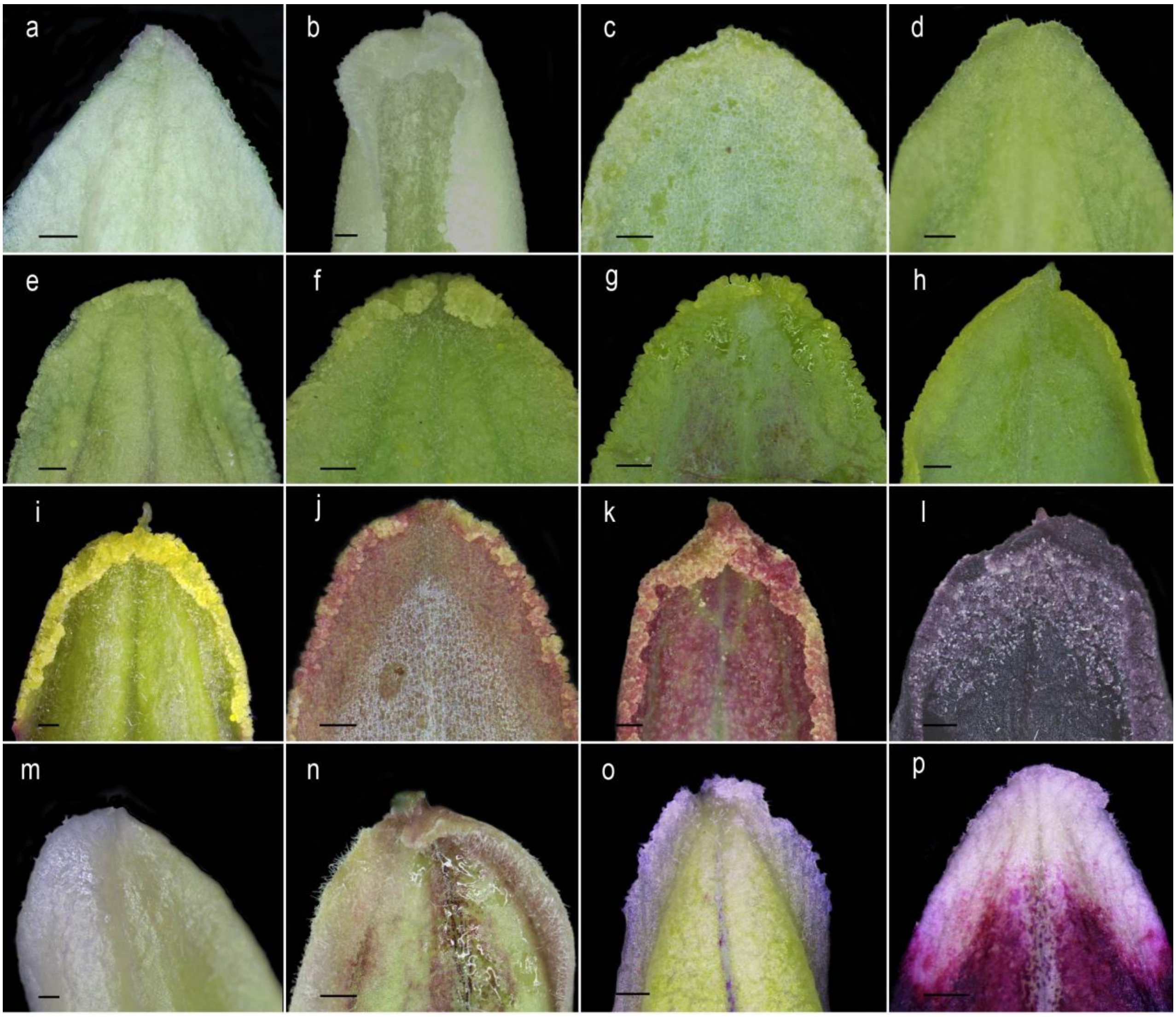

Figure 2 Tepals (ventral side) in *Manihot* species. Note the modified epidermis on tepals margins. **a** *M. tripartita*. **b** *M. sagittata*. **c** *M. debilis*. **d** *M. fruticulosa*. **e** *M. robusta*. **f** *M. purpurea*. **g** *M. oligantha*. **h** *M. minima*. **i** *M. violacea*. **j** *M. pinatiloba*. **k** *M. congesta*. **l** M. *porphyrantha*. **m** *M. gracilis*. **n** *M. esculenta*. **o** *M. glaziovii*. **p** *M. pseudoglaziovii*. Scale bars: (a-c, e-l, n-o) 0.5 mm, (d, m) 0.2 mm, (p) 1 mm.

(small to long) or branching (one to two). Trichomes frequently demonstrated positive reaction with neutral red (**Fig. 3b-c, f, h-i, k-l, n**).

Floral nectaries (FNs) were very constant in their location and general morphology among species (not shown). We found secretion on the bottom of both male and female flowers in all studied species. The nectariferous epidermis appeared compact and smooth under the stereoscopic microscope.

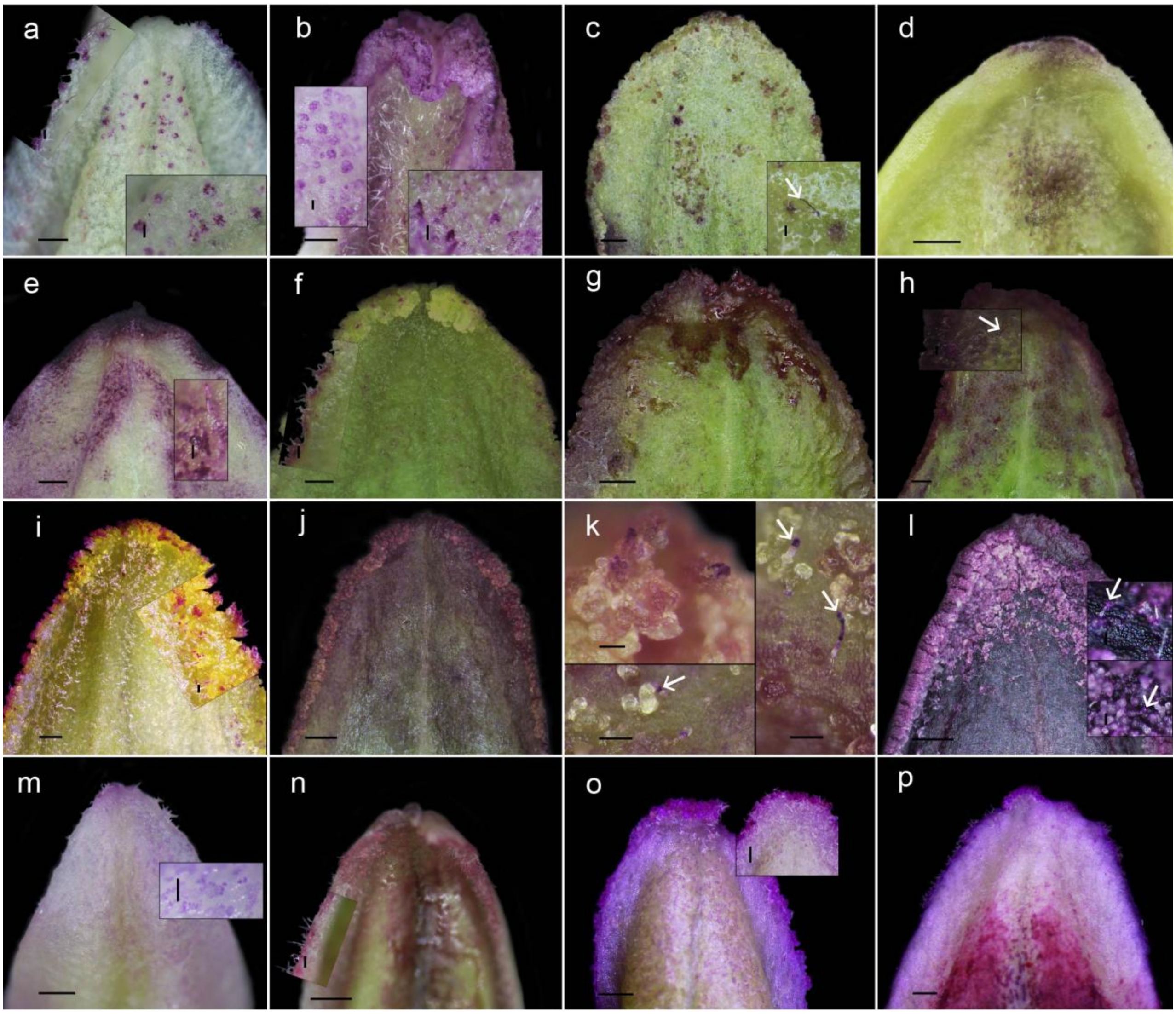

Figure 3 Osmophore regions in ventral side of tepals in *Manihot* species (staining with neutral red). **a** *M. tripartita*, details of tepals margins and central region. **b** *M. sagittata*, reaction in tepal apex, and trichomes. **c** *M. debilis*, detail of patchy epidermis and stained trichome. **d** *M. fruticulosa*, positive reaction for apex and central tepal. **e** *M. robusta*, in detail central tepal with patchy epidermis. **f** *M. purpurea*, note the patchy epidermis (weak reaction) in central of tepal and in detail stained tepals margin. **g** *M. oligantha*. **h** *M. minima*, in detail the reaction of tepals margin next to a stained trichome. **i** *M. violacea*, note in detail the tepals margin positive reaction. **j** *M. pinatiloba*. **k** *M. congesta*, papillae and trichome-like positive reaction. **l** M. *porphyrantha*. **m** *M. gracilis*, note the strongest reaction on tepal apex and in detail the patchy epidermis. **n** *M. esculenta*, in detail tepals margin and trichomes positive reaction. **o** *M. glaziovii*, apex showing the strongest reaction. **p** *M. pseudoglaziovii*, note the reaction for margins. Scale bars: (a-j, l-p) 0.5 mm, (details h-i, o) 0.2 mm, (details a-c, e-f, k-n) 0.1 mm.

Osmophores structure

We confirmed the osmophore occurrence based on the papillose epidermis and ruggae with a great number of secretory vesicles, epidermal cells with lipophilic content and terpenoid

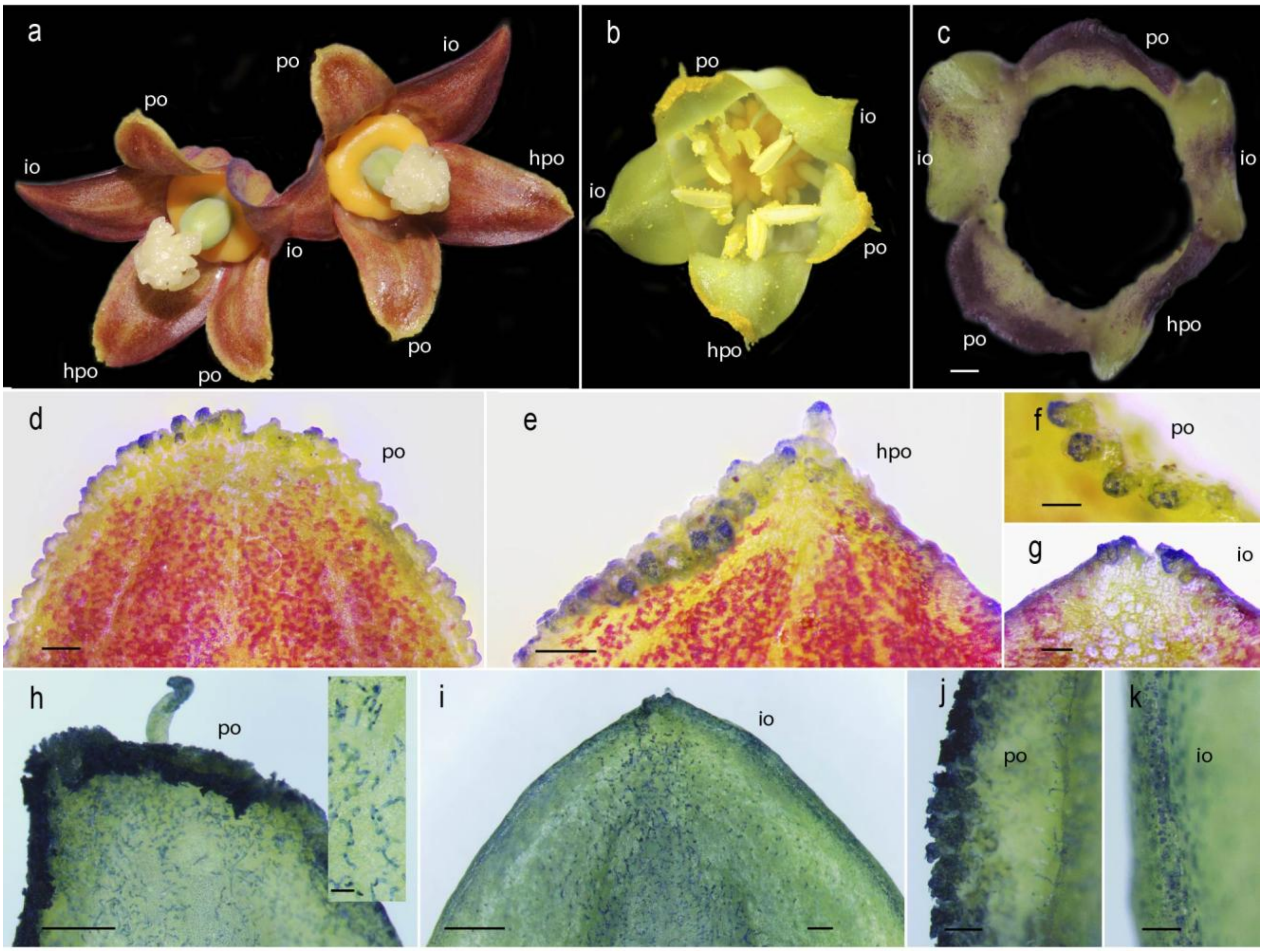

Figure 4 Osmophore pattern on the perianth in *Manihot* species (**c**, neutral red; **d**-**k**, NADI reagent). **a, d-g** *M. oligantha*. **b, h-k** *M. violacea*. **c** *M. fruticulosa*. **a** Note the round yellowish margins of tepals. **b** Proeminent tepals margin also yellowish colored. **c** Pattern on perianth. **d** Positive reaction for terpenes, revealed by NADI reagent (blue coloration) in tepals margins. **e** Tepal (hpo) reacting for terpenes in margins, more proeminent in the left side whereas appears the papillae. **f** Detail of papillae stained by NADI. **g** Tepal apex (io) that demonstrates restricted terpenes content. **h** Tepal showing intense reaction with NADI in margins and also in trichomes (detail). **i** Tepal (io) with the positive reaction for terpenes on margins and central tepal. **j** Strong reaction for terpenes in papillae on the margins of tepal. **k** Margins of tepal (io) showing the smooth surface and diffuse terpene content. Abbreviations: (io) inconspicuous osmophore, (hpo) half prominent osmophore, (po) prominent osmophore. Scale bars: (c) 1 mm, (d, e, j) 0.2 mm, (f, g, detail of i, k) 0.1 mm, (h, i) 0.5 mm.

accumulation under the cuticle. The three species were different in the general osmophore structure but have a similar gland location and in the papillae and trichomes components (**Fig. 5**). The osmophores of *Manihot* show floral position in the apical part of tepals and in the tepals margins. In SEM, the osmophores did not vary between sexes of the species. Cerrado species had epidermis projected clefts on the ventral side of the tepals, formed by the papillae (**Fig. 5i-j, o-p**). *M. violacea*

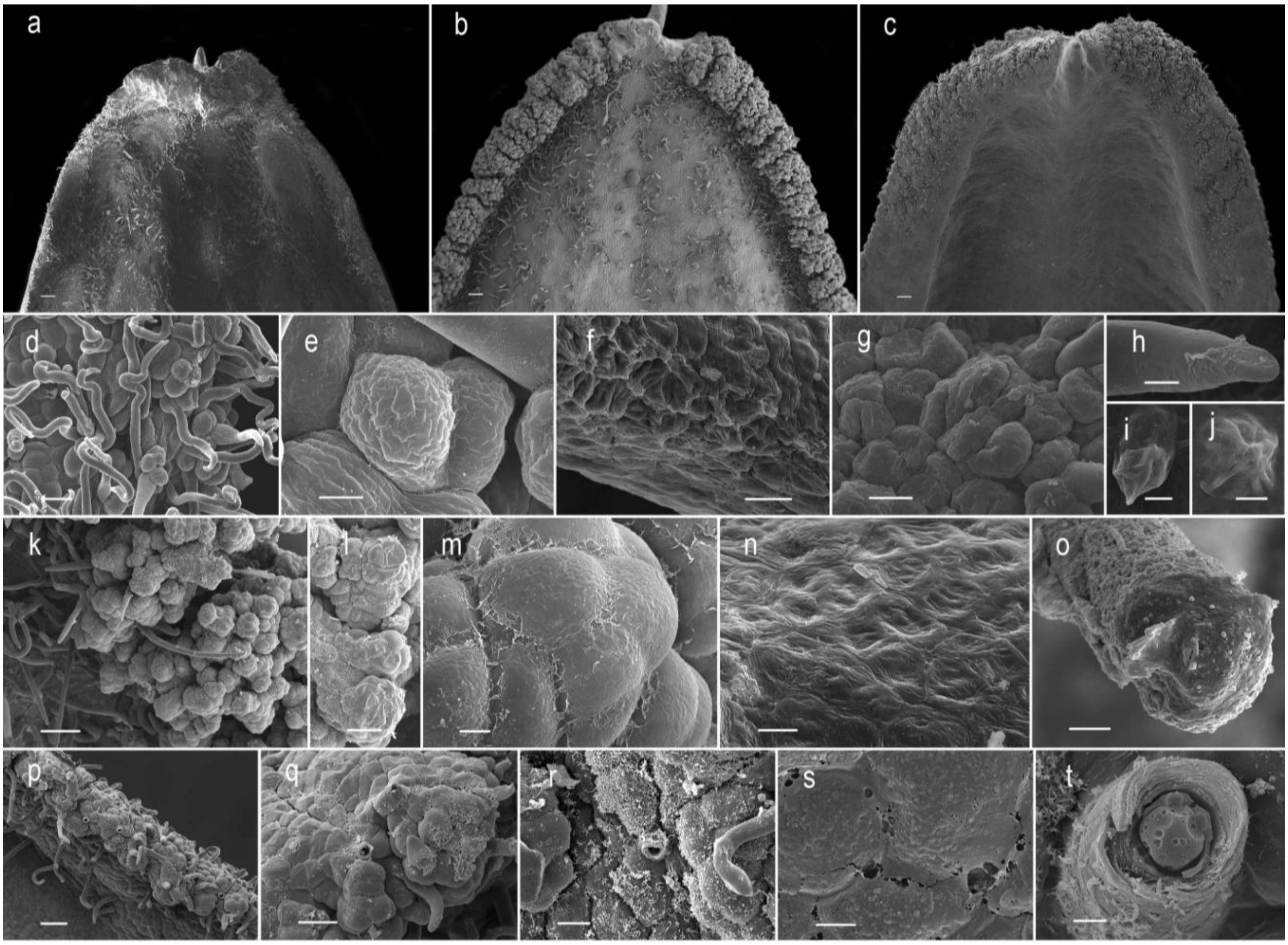

Figure 5 Ventral side of tepal in SEM of the *Manihot* species showing osmophores in apices and margin. **a-h** *M. esculenta*. **i-n** *M. violacea*. **o-t** *M. oligantha*. **a** General aspect of tepal showing many trichomes. **b** Margin of tepal with the projected rounded epidermis (papillae) and trichomes. **c** Tepal's margin with small projections. **d** Tepal's papillae with smooth striation in the cuticle. **e, f** Detail of the projected epidermis of tepals margins with wrinkled cuticle. **g** Unusual epidermal projection in the central tepal. **h** Trichome with peeling. **i** Tepal showing papillae in margins and trichomes nearby it. **j** Papillae with a cavity and trichomes. **k** Papillae with a smooth wrinkled surface. **l** Detail of tepals margin with wrinkled cuticle. **m** Projected epidemis that formed the papillae showing slightly striated surface and cuticle peelings. **n** Trichome with cuticle blisters. **o** Overall tepal morphology, with rugae and small trichomes within margins. **p** Tepal margin showing epidermal projections with small clefts and ruptured trichomes (setae). **q** Detail of epidermal projections, displaying different shapes and a bifurcated structure like-papillae. **r** Detail of epidermis margin with smooth and projected surface and ruptured trichome. **s** Cuticle bearing blisters and peeling. **t** Detail of ruptured trichome containing secretion with circular marks. Scale bars: (a, i, o) 100 µm, (b, c, k) 20 µm, (d, f, g, l, p, q, r) 10 µm, (e, s) 5 µm, (h) 1 µm, (j) 50 µm, (m) 3 µm, (n, t) 2 µm.

had cuticular blisters and slightly rugose surface (**Fig. 5k, m**) while *M. oligantha* appeared with a smooth cuticle that showed spherical marks as well as small projections of vesicles secretion (**Fig. 5q-s**). *M. esculenta* osmophore appeared different, with a compressed aspect, cuticle striation and scattered epidermal projection (**Fig. 5c-f**). The trichomes differed in location and density among species: *M. esculenta* had dense trichomes especially in tepal margin but not in the apex; *M. violacea* had mainly trichomes near the tepal margin, while *M. oligantha* had them mainly in the tepal margin (**Fig. 5a, h-i, n-o, r**). Many trichomes in this last species appeared ruptured (**Fig. 5p, r, t**).

The osmophores inner structure in the three *Manihot* species was formed by secretory cells of the epidermis, cells containing dense vesicles in cytoplasm and trichomes (**Fig. 6**). The secretory epidermal cells of the osmophores have large amounts of vesicles and dense nucleus (**Fig. 6b, d-e, g-h, n**) but their structure lack precise organization which appeared from one-layered to multi-multilayered (**Fig. 6d, f, o**). The osmophore had xylem and phloem vascularization and laticifers appeared at the inner part of the tepal structure. The secretory epidermis was predominantly papillose, though in *M. esculenta* it tended to palisade in rugae, in osmophore apex (**Fig. 6c**).

Since idioblasts can be treated as a specialized cell containing a distinctive form, size and chemical composition as described in De Luca *et al.* (2000) and Castro and Demarco (2008), many cells in *Manihot* floral glands bear those features and are considered here as idioblasts. Additionally, the idioblasts are a common feature in Euphorbiaceae, occurring in several plants tissues (Metcalfe and Chalk 1950), including in *Manihot* leaves and fruits (Oliveira and Oliveira 2009, da Cunha Neto *et al.* 2014). The anatomy revealed a great number of idioblasts in *Manihot* tepals and the secretory cells of osmophores epidermis often surrounded idioblasts, especially in *M. oligantha* and *M. violacea* in the papillae (**Fig. 6f, j, l, m, o**). Besides that, *M. violacea* had trichomes-like structures formed by idioblasts or secretory epidermal cells (**Fig. 6j, k**). The trichomes were mainly uniseriate but could also be composed of two cells (**Fig. 6j, p**).

Floral nectary structure

The structure of FN was conservative for the three *Manihot* species (**Fig. 7**). The morphology of FNs was discoid, the male flower had FN with 10 lobes, whereas female flower had an entirely round nectary, but commonly presented reentrances or lobes in nectary, occasionally with staminate

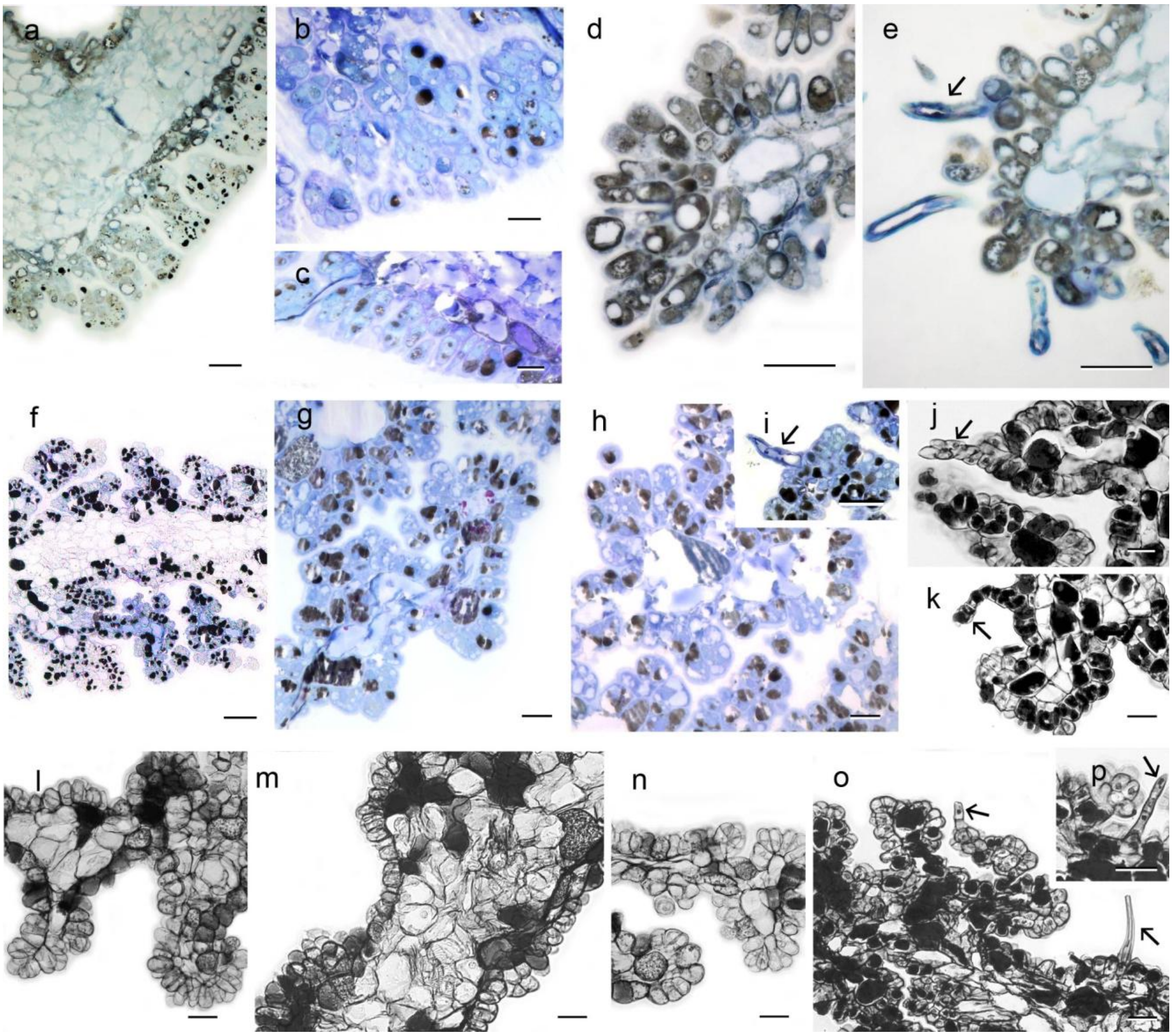

Figure 6 Anatomy of the osmophore in the tepal margins of *Manihot* species (stains: a-i toluidine blue 1%, j-p safranin-toluidine blue). **a-e** *M. esculenta*. **f-j** *M. violacea*. **k-o** *M. oligantha*. **a** Tepal apex with osmophore forming projections in the ventral side. **b** Osmophore papillae, note the cells with many vesicles. **c** Osmophore epidermis, composed of secretoty cells and idioblasts. **d** epidermal projection constituted mainly by idioblasts. **e** Trichomes (setae) in epidermal projection. **f** Osmophore apex showing papillae with longer extend ventrally. **g** Secretory cells of epidermis and idioblasts, note that epidermis can be projected by idioblasts. **h** Osmophore epidermis, with a large amount of vesicles and an idioblast appear in degradation centrally. **i** Detail of trichome (setae) in osmophore epidermis. **j** Secretory cells of osmophores forming a projection similar to trichomes (setae). **k** Idioblasts with dense content also forming a structure similar to trichome (setae). **l**, **m, n** Papillae with longer extend ventrally and large idioblasts. **o, p** Epidermal projections with idioblasts and secretory cells, note the different trichomes (setae). Scale bars: (**a, b, d, e, f, i**) 50µm, (**c, g, h, j, k, l, m, n, o, p**) 30µm.

vestiges (**Fig. 7a-b**). In SEM, FNs presented compact epidermis with cells of variable shape, often covered by a substance, maybe derivate from the cuticle (**Fig. 7e-h**). Sparse stomata appeared in mid-down position, near wrinkled areas (**Fig. 7c-d, i**).

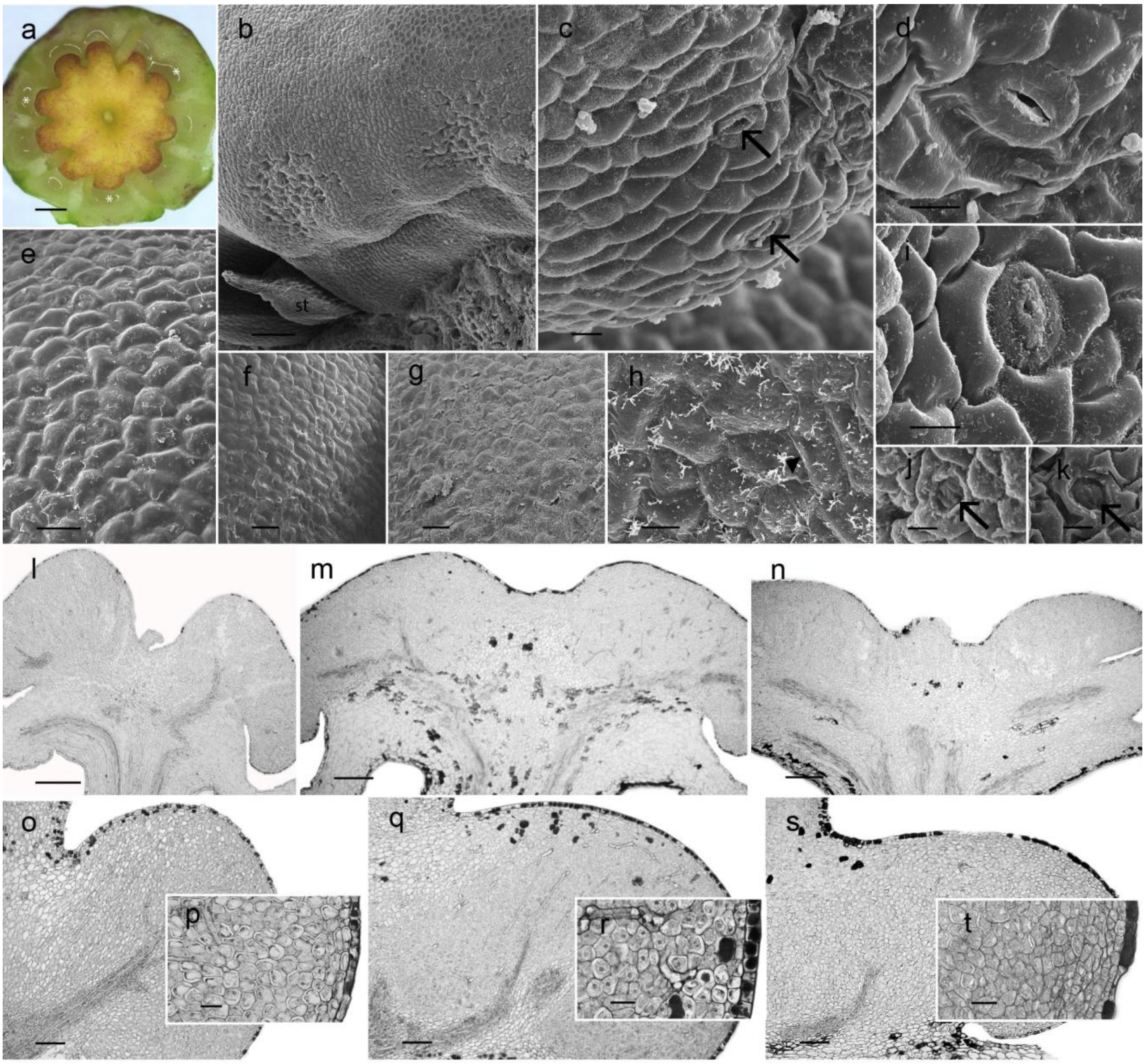

Figure 7 Floral nectaries in *Manihot* species – morphology (a), micromorphology (SEM, b-k) and anatomy (l-t). **a, e, l, o-p** *M. esculenta*. **b-d, g-k, m, q-r** *M. violacea*. **f, n, s-t** *M. oligantha*. **a** Nectary of male flower, note the shining nectar that accumulated on the bottom (asterisk). **b** Lower view of nectary with wrinkling areas and a staminoide. **c**, **d** Detail of the previous photo, showing modified stomatas (setae). **e, f, g** Epidermis of nectary, showing shrink cells. **h** Epidermis with stigmatic secretion (triangle) from the female flower. **i** modified stomata with pore occlusion in *M. oligantha*. **j, k** detail of not functional stomatas. **l, m, n** Longitudinal section of male flowers with vascularized nectary except for the *M. oligantha*. **o, p, q** Nectary of female flowers in longitudinal section, note the laticifers spread along nectariferous parenchyma. **r, s, t** Detail from previous sections showing the uniseriate epidermis formed by idioblasts and nectariferous epidermal cells. Scale bars: (a) 1 mm, (b) 100 µm, (c, d, h, i, j, k, o, p, q) 10 µm, (e, f) 30 µm, (g) 20 µm, (l, m, n) 300 µm, (r, s, t) 25 µm.

The internal structure was formed by a nectariferous epidermis single-layered composed of densely stained idioblasts (**Fig. 7l-t**). These idioblast frequency changes in species, being more recurrent in *M. violacea*, frequent in *M. oligantha* and less frequent in *M. esculenta*. The epidermal cells varied in shape from cubical to rectangular and had a relatively thick cell wall. Nectariferous parenchyma was multilayered and specialized, composed of small secretory cells with a conspicuous nuclei and dense granular cytoplasm, characteristic of floral nectaries (Fahn 1988). FN was vascularized mainly by phloem but also xylem, and vessels had an origin in the receptacle (**Fig. 7l-m**). We observed 10 traces that ascended to nectary, as earlier described in floral anatomy of *M. esculenta* (Venkata-Rao and Ramalakshmi 1968). Male flowers of *M. oligantha* were supplied by those vessels, although it was the only floral type that we did not find vascularization directly in nectariferous parenchyma (**Fig. 7m**). Laticifers were spread along all FNs and the majority was branched, not articulate.

Floral glands histochemistry

The main chemical compounds found in floral glands did not differ in *Manihot* species (**Table 2**), and between female and male flowers. Terpenes were abundant for both floral glands and all structures had blue coloration indicating essential oils: osmophores, its trichomes, FNs, all had vesicles, whereas idioblasts could present dense homogenous stain (**Fig. 8a-c, e, t**). We found similar results for NADI (terpenoid reagent) and neutral red. *Manihot esculenta* had a very strong reaction with NADI (not shown), while *M. violacea* was moderate and *M. oligantha* was less accentuated. The reaction for lipids was more subtle, and occurred in epidermal cells of osmophores and its trichomes (**Fig. 8d, f-i**) and less often in idioblasts of the glands (**Fig. 8i, k**). Secretory idioblasts also were positive for phenolic compounds in both glands (**Fig. 8e, j, p, q, t**).

FNs were positive for starch and carbohydrates that appeared abundant in the parenchyma cells, and only *Manihot esculenta* presented starch in the nectariferous epidermis, though occasionally (**Fig. 8k-l**). Depending on the stage of secretion in FNs, the quantity of starch varied. In the beginning, it was abundant, with grains all over the cytoplasm of nectariferous parenchyma cells,

Table 2 Histochemical reactions in the tissues of the floral glands in three *Manihot* species

Floral gland and histochemical test	Compound	Secretory structures and species											
		M. esculenta				*M. violacea*				*M. oligantha*			
Osmophore		Ep	Tr	Id		Ep	Tr	Id		Ep	Tr	Id	
Lugol	Starch	-	-	-		-	-	-		-	-	-	
Sudan Black	Lipids	+	-	+		+	+	+		+	-	+	
Sudan IV	Lipids	+	-	-		+	-	-		+	-	-	
Ferric chloride III	Phenolic compounds	-	-	+		-	-	+		-	-	+	
NADI	Terpenoids	+	+	+		+	+	+		+	+	+	
Floral nectary		Ep	Pa	Id	Lat	Ep	Pa	Id	Lat	Ep	Pa	Id	Lat
Lugol	Starch	+	+	-	-	-	+	-	-	-	+	-	-
PAS	Carbohydrates	+	+	+	+	+	+	+	+	+	+	+	+
Ruthenium red	Acidic mucilages	+	-	-	-	+	-	-	-	+	-	-	-
Sudan Black	Lipids	-	-	+	-	-	-	+	-	-	-	+	-
Ferric chloride III	Phenolic compounds	-	-	+	-	-	-	+	-	-	-	+	-
Wagner's reagent	Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+
Comassie R250	Proteins	+	+	+	+	+	+	+	+	+	+	+	+
NADI	Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+

Notes: (+ positive; - negative). Abbreviations: Ep, epidermis; Tr, trichomes; Id, idioblasts; Pa, parenchyma; Lat, laticifers.

and on more advanced stages there was much less starch. Acid mucilage was detected in cells walls of the nectariferous epidermis (**Fig. 8o**). In all FNs tissues, we detected alkaloids and proteins, with an abundance of alkaloids (**Fig. 8r, s**).

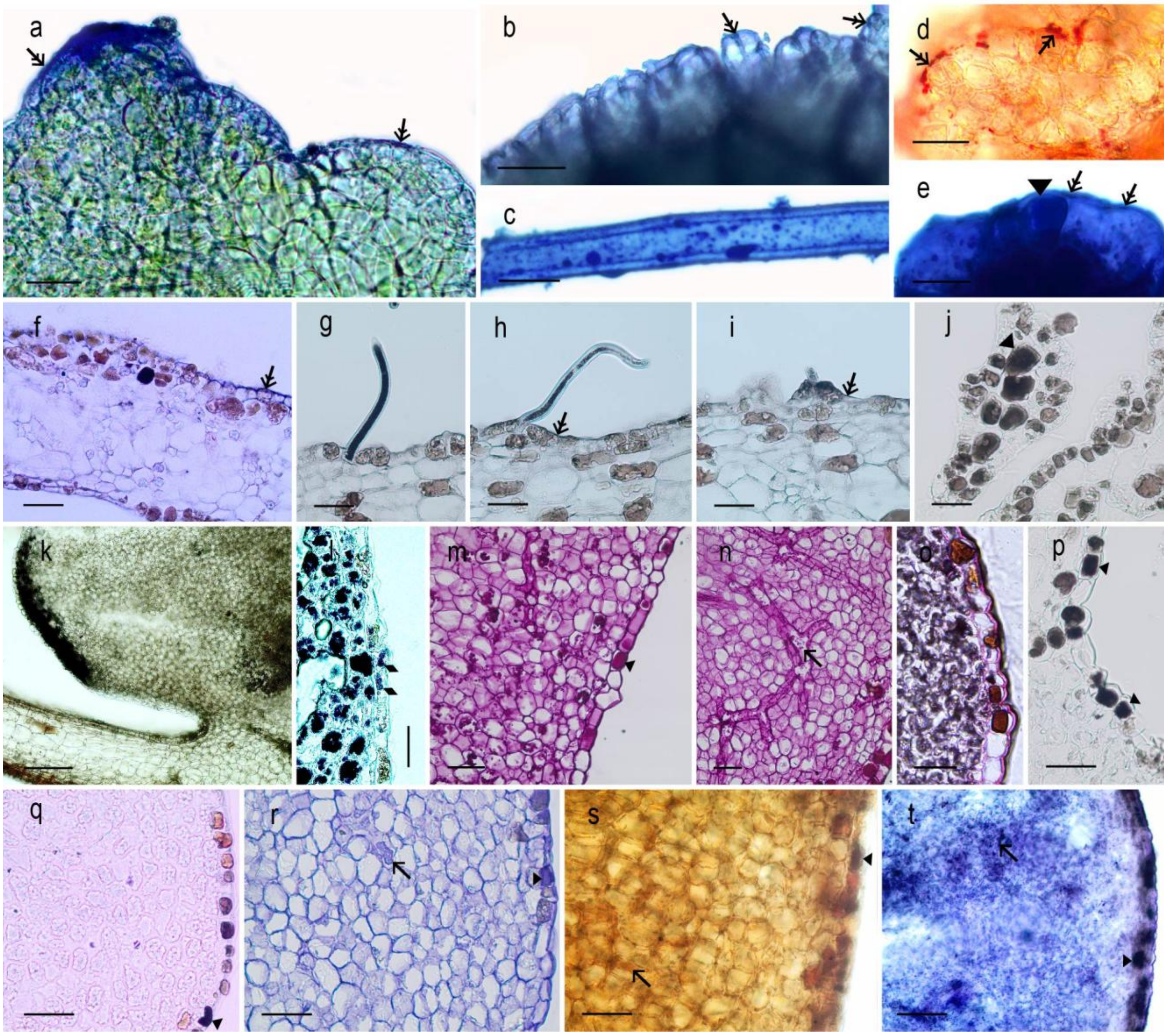

Figure 8 Histochemical reactions of the floral glands of three *Manihot* species for osmophores (**a-j**) and FN (**k-t**). **a, i-k, m, r** *M. oligantha*. **b-c, f, l, p, s** *M. esculenta*. **d-e, g-h, n-o, q, t** *M. violacea*. **a** Terpenes (blue coloration) in tepal apex, double-arrow pointing secreted terpene that accumulates below the cuticle. **b** Margin of tepal bearing papillate epidermis with secreted terpenes. **c** Trichome showing terpene content. **d** Papillate epidermis with lipophilic content (red) pointed by double-arrow. **e** Papillae of the epidermis (double-arrow) and idioblast (triangle) positive for terpene. **f** Secretory epidermis containing lipids (dark) (double-arrow). **g, h, i** lipophilic content (dark) in uniseriate trichomes and also epidermis (double-arrow). **j** Idioblasts (triangle) with phenolic compounds (dark-brown). **k** Nectariferous parenchyma with starch (dark) near the epidermis. **l** Nectariferous epidermis positive for starch (losangle). **m, n** Nectariferous parenchyma, idioblasts (triangle) and laticifers (arrow) with positive reaction for carbohydrates (pink). **o** The cuticle of the nectariferous epidermis with relatively thick cuticle containing acid mucilage (pink). **p** Idioblasts (triangle) of nectariferous epidermis reacted positively for phenolic contents (dark-brown). **q** Discreet reaction of idioblast (triangle) from the nectariferous epidermis for lipid (dark). **r** Laticifer (arrow), idioblast (triangle) and general reaction for protein detection (blue). **s** Positive reaction for alkaloids (reddish-brown) in nectariferous tissue. **t** Nectary containing terpenes (blue) in epidermis and parenchyma. Scale bars: (a, d) 25 µm, (b) 100 µm, (c, e, f, g, h, i, j, l, m, n, o, p, q, r, s, t) 30 µm, (k) 200 µm.

DISCUSSION

Osmophores in Manihot species

Osmophores can be quite common among flowering plants, and are a conspicuous feature in the flowers of all studied *Manihot*. Though, our new finding for the genus is the second register of this gland for Euphorbiaceae. The study of Gagliardi *et al.* (2018) registered osmophores in the apex of petals in *Joannesia*. Here, the osmophores occur in ventral tepals, especially in apex, which is common as described by Vogel (1990); besides, the tepals margins also featured the gland. Although neutral red can fail to identify osmophore in certain cases (Kearns and Inouye 1994, Gonçalves-Souza *et al.* 2017), the scent-producing tissues, rich in lipophilic volatile oils, can efficiently react with neutral red, as observed here. The terpenes appeared as the main compound in *Manihot* osmophores. Floral volatiles is diverse (Jürgens *et al.* 2008), but terpenes can comprise most of its diversity, being a very common constituent (Dudareva and Pichersky 2000, Knudsen *et al.* 2006, Borghi *et al.* 2017).

The osmophores positioning on *Manihot* tepals are very conservative for the species and forms a pattern on perianth: margin conspicuous always next to the discreet one. Corolla and/or calyx have mainly two roles: attraction and defense (Delph 1996, Galen 1999, Endress 2010). Likely due to the reduction of *Manihot* floral whorls, its perianth could do both functions. The osmophore as a chemical barrier for pathogens was observed in a citrus flower (Marques *et al.* 2015). These researchers also found that floral buds had compact papillae that could represent a structural barrier for antagonists, preventing infection by a pathogen fungus. Here, the papillae on tepals margins in contact with the other inconspicuous side of tepals margins could also function as a structural barrier. In addition, cells related to defense are in those papillae, such as idioblasts that could protect the floral structure against pathogens and florivory; this protection role is recognized for idioblasts, and is reinforced by other structures found is osmophores such as laticifers, trichomes, and also for the detected substances (e.g phenolic compounds and terpenes) (Mura *et al* 2005, Hagel *et al*. 2008, Demarco and Carmello-Guerreiro 2011, Mithöfer and Boland 2012, Pichersky and Raguso 2016). At anthesis time, the idioblasts have its content mobilized in osmophores, similarly with what was found in Leguminosae (Marinho *et al.* 2018). However, as demonstrated by these authors, the idioblasts mobilization was not involved in scent production and otherwise could act in plant protection. In short, the consistent patterns of osmophores on *Manihot* perianth, the identified compounds, and structures, all suggest a pronounced defense function by this floral gland.

The internal structure of osmophore is variable despite the constancy in its perianth position and secretory elements. The variation includes that in the same region the secretion process can be different for each cell: some cells appear with a high amount of vesicles in the cytoplasm, others with vesicles merging with cell walls and other with no vesicles at all. Even the stain differs in a sample, to papillae from another. We might attribute the different secretory stages with a continuum emission of volatiles. The liberation of volatiles by osmophores was described to occur through epidermis cuticle that should be facilitated by cuticle striation (Stern *et al.* 1987, Vogel 1990, Marinho *et al.* 2014), but other ways to expose the secretion were reported as stomata (de Melo *et al* 2010), trichomes diffusion (Wiemer *et al.* 2009), cuticular pores (Pridgeon and Stern 1983) and cuticular blisters rupture (Sazima *et al.* 1993). In addition, volatilization by passive diffusion is not possible and the emission process appears complex and challenging to prove (Widhalm *et al.* 2015, Borghi *et al.* 2017). Volatile emission is related to increases in the epidermal surface (as papillae, rugae) or to thermogenesis processes (Raguso and Gottsberger 2017, Vogel 1962). In our samples, *M. esculenta* was the only one with cuticle striation. The epidermis presented cuticle expansion detected solely in *M. violacea*, likely due to secretion diffusion, while *M. oligantha* presented cuticle blisters. Despite the differences, all of them have enlarged areas in tepals margins that should increase scent emission.

Secretory trichomes appear to be very common in tepals of *Manihot* species. Trichomes can emit floral volatiles (Wiemer 2009), even though their content was showed to not contribute to floral scent and rather act in flower defense (Effmert *et al.* 2005). In our samples, they seemed to work together with epidermis for terpene emission, but they also work independently. It is known that the production of conical cells in petals of *Antirrhinum* (Plantaginaceae) has its pathway in common with the trichomes (Glover *et al.* 1998), therefore the epidermis and trichomes could be connected in its development. The trichome-like papillae could function as the epidermis and emit terpenoids, whereas the uniseriate trichome (more differentiated) might function for deterrence. Although, *M. violacea* species has uniseriate trichome that appears non-glandular fullith lipophilic content and terpenes and *M. esculenta* trichomes are also rich in terpenes. A connection between these trichomes and the secretory epidermis is likely, but the function of these former structures is still unclear.

Floral nectaries in Manihot species

The floral nectaries of the studied *Manihot* species are highly conservative in its structure, secretory components, and chemical compounds. The epidermis has few modified stomata located whereby nectar accumulates, suggesting that stomata secrete nectar. Despite that, wrinkled areas occur in floral nectary during anthesis, at the same time the epidermis appear narrower and can be degenerated. Due to the scattered stomata and epidermal modification after secretion, probably nectariferous epidermis also releases nectar. It is known that nectar secretion by stomata can be a non regulated process (Razem and Davis 1999, Wist and Davis 2006), even though several authors attributed for modified stomata the nectar release (Davies *et al.* 2005, Paiva and Machado 2008, Stpiczynska *et al*. 2014), including other Crotonoideae (Euphorbiaceae) as *Joannesia* and *Croton* (Gagliardi *et al.* 2016, Feio *et al.* 2016). Similarly with those in Crotonoideae, in *Manihot* species, floral nectary has single-layered nectary epidermis, but *Croton* did not present differentiated epidermal cells strongly stained and *Joannesia* contained chlorophyll in nectariferous parenchyma, which differentiates *Manihot* nectary.

Related to its origin, nectar can be secreted in small quantities at a time (chlorophyll) or rapidly when starch is stored in the parenchyma, often its storage being source to nectar syntheses

(Pacini *et al*. 2003, Razem and Davis 1999, Durkee *et al*. 1981). The *Manihot* species floral nectaries largely contain starch grains that disappear depending on the stage of secretion. In field observation, nectar release starts hours before flower opening, despite that we only observed floral visitors attracted to the floral bud near to its opening. The process indicates that the starch is stored in the parenchyma, working as the precursor of nectar, then, in pre-anthetic flowers occur the nectar release and during floral anthesis, the nectar metabolism slows down. Flower of *M. esculenta* was reported to last one day (Halsey *et al*. 2008). Similarly, the nectar strategy of *Manihot* species appears to be ephemeral, consisting of offering this reward already when the flower open for the first time, and afterward little or no nectar should be produced.

Many substances related to plant defense are found in nectary tissue of *Manihot* species, such as phenolics, alkaloids, and terpenes (review in Mithöfer and Boland 2012). Cassava stems, roots and leaves contain these mentioned compounds that can act in the defense system (Blagbrough *et al.* 2010, Thiyagarajan and Suriyavathana 2010, Zeng *et al.* 2015). In plants damaged by herbivory, the alkaloid levels are positively correlated between nectar and leaves (Adler *et al*. 2006). Besides the protection function, alkaloids in nectar might affect pollination, including repel illegitimate visitors and shape the dynamics of pollinators visits, promoting outcrossing (Kessler and Baldwin 2007, Kessler *et al.* 2008, Irwin and Adler 2008). It is likely that nectar in *Manihot* contains alkaloids that could enhance reproductive fitness and flower defense.

Floral diversification in Manihot species and evolutionary implications

As discussed previously, osmophore seems to have a dual role of protection and attraction due to its structure and compounds. Plant defense system often evolved first, with a floral attractant or reward evolving secondarily, as suggested for monoterpenes, resins, fragrance and inflorescence color (Pellmyrl and Thien 1986, Armbruster *et al.* 2009, Hanley *et al.* 2009, Schiestl 2010). Despite, evolutionary switches in function occurred for triterpene resin, which started as a toxin and deterrent compound, switching to an attractive floral reward and then turning back for the protection role (Armbruster *et al.* 1997). In addition, modifying the concentration of terpenes may shift its functions (Theis and Lerdau 2003) or terpenoids compounds can even have a double function of attraction and defense (Knudsen *et al.* 2006). These findings all indicate a lability of terpenes. Here, we implied that the osmophore first evolved for defense due to antagonist pressure and, afterward, could function in pollinator advertisement by the emission of essential oils (terpenes).

From osmophore consistent pattern, we also propose that its disposal has an economic value and would be a sort of cheaper investment. Considering that the genus is predominantly monoecious and depends on animal pollination (Alves 2002), reproductive features could be related to certain constraints of this sexual system. It is known that it is more costly to produce unisexual flowers (Jong *et al.* 2008), this might explain why the genus flowers are generally small sized and have perianth in a single whorl (least costly). Taxa bearing unisexual flowers are predicted to have generalist pollination (Bawa 1980, Charlesworth 1993, Jong *et al.* 2008). In fact, the morphology of both male and female flowers in *Manihot* is among the highest generalization level and enables most visiting animals (Olesen *et al.* 2007). The sexual dimorphism is predominantly in the genus, and the tendency is the perianth length in male flowers being bigger than female flowers. It is known that female flowers reduced size is relative to the hermaphroditic ancestor of related groups on *Lycium* (Miller and Venable 2003). Allocation of resources to male and female functions is related to sexual selection (Willson 1979) and a smaller female might save resources to allocate in fruit development (Huang *et al.* 2006).

For bracts features an economy seems to exist as well, especially in the narrow endemic species that most have deciduous or very inconspicuous bracts (e.g. *M. oligantha*). Lavergne *et al.* (2004) explain in their work on the Mediterranean region that narrow endemics invest less in reproduction than its widespread congeners due to the need for local persistence on the occupation of rocky soils. Here, the narrow studied species often occur in this soil condition (see Mendoza *et al.*

2015, 2016) and deciduous bracts might save resources. It is known that bracts have both attraction and defense function (Sun *et al.* 2008, Armbruster *et al.* 2009, Endress 2010). For the narrow endemic species is more likely that these structures protect the buds than function in attractiveness. If showy, the bract of *Manihot* could act in pollinator attraction, even though few species here presents attractive bracts (*M. purpurea, M. tripartita, M. violacea*). As a result, the perianth should often be the major verticil with attraction function in species.

In *Manihot* genus the male flowers are campanulate or tubular-campanulate shape, whereas the female flowers have free tepals and are wide-opening (see Rogers and Appan 1973). Differently, from eight species restricted distribution in the Cerrado, six had the female flowers with connected tepals, comparing with the wider-distributed species, none have this feature. The female flowers of restricted endemic species also show the smallest flowers. It is known that changes in flower size can correspond to changes in the members' size of a functional group that pollinates the flowers (Galen 1999, Fenster *et al.* 2004). We suggest that the Cerrado species evolved differential floral traits revealed by the morphology of the female flowers, which might be adapted to their local pollinator fauna, thus indicating more specialization of species on this biome.

Furthermore, specialized morphology of osmophores occurs in Cerrado taxa, wherein it can be very similar to pollen (Fig. 4b), and actually, bees are attracted to the structure and scrape it (personal observations in *M. violacea*). The reported pollinators for *Manihot* (Nassar and Carvalho 1990) are mainly generalist social bees, in which the learning of flower signals is potentialized by flower odors (Kunze and Gumbert 2001, Leonard 2011). Even generalist pollinators might exert divergent selection in flower shape, in a population under strong selective regimes (Gómez 2009). *Manihot* species have a noteworthy geographic clustering in drier climates, but considering the efforts, its phylogeny is still not well resolved, probably due to its plasticity, ancestral polymorphism, and ongoing speciation (Chacón *et al.* 2008, Duputié *et al.* 2011, Cervantes-Alcayde *et al.* 2015). Despite that, our analyzed species with showy osmophores occurs in subclades of Cerrado (Mendoza

2018, unpublished phylogeny). In contrast, the species that are more widely distributed and the ones from Caatinga biome bear inconspicuous osmophores. Therefore, indicating that cerrado species diversified in its glands.

Although certain floral traits appear constant such as perianth in a single whorl, nectary and osmophores position, the individual variation of flower color, glands morphology and trichomes are characteristic of *Manihot* species, which we could imply phenotypic plasticity of the genus. By plasticity, the conservative flowers may respond to the environment and monoecy constraints. The floral glands in *Manihot* and its secondary compounds could play an important adaptive role since perianth structure kept constant in whorls and tepal number. However, a full analysis of the secretory process and floral volatiles still need to be conducted. We emphasized that the attraction or deterrent effect are closely related and may overlap functions.

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CHAPTER 2

Ecological generalized but not so much: pollinator filtering in monoecious *Manihot* (Euphorbiaceae)

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ABSTRACT

Starting from the available fauna of floral visitors to them performing as pollinators, several floral filters might act. Here, we questioned if the unisexual flowers can function as filter, wherein visitors go only for one floral type. Besides, we reported for the first time the pollination and breeding system of wild *Manihot* in its natural environment. We studied two *Manihot* species, and for each one recorded the visitors and pollinators in two flowering events. We evaluated differences among floral sexes such as floral anthesis and display, floral morphometrics, nectar volume and concentration; also, we conducted hand-pollination treatments. Overall, the *Manihot* species were generalist and attracted several groups of insect visitors, but less were recorded pollinating. *Manihot violacea* showed ecological specialization with bees, whereas *M. oligantha* had two main functional groups (i.e. ants and flies). The floral display sex ratio was male-biased and the functional groups that are low abundant tend to not visit female flowers. Despite, there was a tendency of the female flower being more attractive in nectar traits and it was not dicriminated by pollinators. The *Manihot* species depended on animal pollination to produce seeds and the relative abundance of pollinators contributes to its importance and possible selection of floral phenotypes. Each species have distinctive traits in nectar, floral anthesis, and display that might restrict pollinators. Moreover, the female flowers exert filtering in occasional visitors by its lower number in these monoecy systems. The findings reinforce that unspecialized flowers can restrict pollinators in different ways than floral morphology.

Key words actinomorphic flower, cassava wild relatives, cerrado, endemic species, female-biased nectar, pollination syndromes, sexual dimorphism

INTRODUCTION

In pollination systems, specialization and generalization is a currently debated topic (Brosi 2016). For instance, the concepts of pollination syndrome are being criticized and its predictions have been put to test in nature, yet, evidence seems to support the syndromes overarching patterns and predictions (Wilson *et al.* 2004, Martén-Rodríguez *et al.* 2010, Padyšáková *et al.* 2013, Rosas-Guerrero *et al.* 2014, Abrahamczyk *et al.* 2017, Wilson *et al.* 2017). Simultaneously, generalist interactions plant-pollinators are suggested to be a broad pattern (Waser *et al.* 1996, Lázaro *et al.* 2008, Ollerton *et al.* 2009, Reverté *et al.* 2016). Despite the controversies, it is well accepted that the diversity on plants of these systems ranges from extreme specialized to supergeneralist, in a complex continuum (Waser *et al.* 1996, Johnson and Steiner 2000, Joly *et al.* 2018). Moreover, a different pollination system might be attributed, depending on the scale (time and space), functional category (functional or taxonomic groups) or even methodology (Fenster *et al.* 2004, Petanidou *et al.* 2008, Li and Huang 2009, Ollerton *et al.* 2015).

It seems to exist a dichotomy between specialist—generalist pollination, e.g.: actinomorphic flowers are associated with generalist pollination, whereas zygomorphic flowers are thought to be specialized (Neal *et al.* 1998, Fenster *et al.* 2004, Sargent *et al.* 2004, Gong and Huang 2009). Many authors (cited above) argue that flowers with restricted access could be more specialized than wide open flowers. Nevertheless, the openness of a flower has not shown to be correlated to generalization levels (Herrera 1988, Olesen *et al.* 2007). In addition, other studies suggest that actinomorphic flowers might be more specialized than zygomorphic depending on the community context (Lázaro *et al.* 2008) and that symmetry may not be related to degrees of specialization (Ashworth *et al.* 2015).

Floral morphology that restricts visitor access is not the only indicator of specialization in flowers. In flowers with open rewards, specialization can occur by other floral traits, such as nectar composition that filter certain floral visitors (Johnson *et al.* 2006, Herrera *et al.* 2010), selective olfactory cues (Shuttleworth and Johnson 2010, Burger *et al.* 2017), nectary marks (Wang *et al.* 2018), and anthesis phenology (Martén-Rodríguez *et al.* 2009). In plants that feature separated male and female phases, filtering of potential pollinators might occur, wherein the pollinator needs to visit both phases to deposit pollen in the stigma (Armbruster 2017). Consequently, in a monoecy system, the pollinator should visit both male and female flowers. Furthermore, it is known that female flowers must be discriminated by pollinators (Charlesworth 1993, Ashman 2000, Totland 2004, Etten and Chang 2014), thus, these floral types might act as a floral filter.

According to Rosas-Guerrero *et al.* (2014) findings, floral syndromes were better predicted in tropical plants and in taxa that rely on animal pollination for reproduction (monoecious, dioecious, or self-incompatible systems). *Manihot* is a neotropical genus, predominantly monoecious and outcrossing system (Rogers and Appan 1973, Da Silva *et al.* 2003, Lai *et al.* 2014). Therefore, in *Manihot* syndromes or specialization is expected. The *Manihot* flowers are actinomorphic with exposed nectary that appears to offer no barrier for visitors, and the only reported pollinators are hymenopterans, among eusocial wasps and bees (Faegri and Pijl 1979, Kawano 1980, Rogers and Appan 1973, Nassar and Carvalho 1990); however, most pollination studies of the genus were in cultivars. Even though there is an urgency to conserve crop wild relatives due to its possible contributions to crop improvement and helping global food security (Vincent *et al*. 2013), e.g. the wild species *M. oligantha* have great potential to increase protein content through hybrids with cassava (Nassar *et al*. 2012), the pollination system of wild *Manihot* species remains unexplored.

Here, we studied two monoecious species of *Manihot* with the following objectives: (1) evaluate the specialization-generalization of the pollination system concerning the array of floral visitors, pollinators and functional groups; (2) verify the dependence on animal pollination and the existence of pollen limitation by exploring reproductive traits of breeding systems; (3) investigate if monoecy could act as a filter for pollinators. For this, we studied floral traits related to pollinator filtering such as differences in floral anthesis and display, rewards and morphology in male and female flowers.

Furthermore, we checked functional groups that visited only one floral type or did not contact reproductive verticils of both male and female flowers, thus resulting in filtering.

MATERIAL AND METHODS

Study species and populations

We studied two species of *Manihot* endemic to Brazilian Cerrado: *M. violacea* Pohl and *M. oligantha* Pax. The main center of diversity and endemism of the genus is in Cerrado, therein *Manihot* species often present narrow distribution in this biome (Rogers and Appan 1973, Simon *et al.* 2018). The primary site that occurs *M. oligantha* is in the Private Reserve of Natural Heritage Serra dos Topazios (-16.737399, -47.689693), Cristalina-GO. The vegetation has predominately presence of herbaceous vegetation, subshrubs, and spaced trees. For *M. violacea* we assessed two localities, in Brasilia-DF, Dom Bosco Ecological Park (-15.796089, -47.805440) and a slope Cerrado area popular named ‘Córrego do Urubu’ (-15.7182440, -47.8518380). These areas have vegetation which mostly consists of Cerrado *sensu stricto* with shrubs, herbaceous and trees.

The *Manihot* flowers are conservative with single whorl in pentamerous perianth and have protogyny at the inflorescence level (Rogers and Appan 1973). The two studied species differ in plant size length, inflorescence traits, bracts, and flower color. *Manihot violacea* is a subshrub ca. 1.0 m tall, with greenish flowers and numerous conspicuous foliaceous bracts that form a compact inflorescence (**Figure 1A-C**). The second, *M. oligantha* is nearly acaulescent subshrub ca 10 cm tall, with purplish flowers, inconspicuous setaceous bracts and inflorescences that are elevated above the vegetative part (**Fig. 1D-F**). Both species present strong floral dimorphism, with polytepalous female flowers (FF) and gamotepalous male flowers (MF).

Floral anthesis and display

Figure 1 The studied *Manihot* species showing their habitat, inflorescences, and unisexual flowers. A-C, *M. violacea*, and D-F, *M. oligantha*.

We followed the floral anthesis for 30 marked buds in pre-anthesis, for each flower sex, in a period of three consecutive days. The presence of floral rewards (pollen, nectar) and floral movements (anthers contraction and retraction, tepals positioning) helped us for flower functioning evaluation. For daily floral display, we counted the total number of male and female flowers (Yakimowski *et al.* 2011). For this, we did preliminary phenological observations and established 30 random individuals for samplings, and assessed the population over one year to record a flowering season (May 2017 to April 2018).

Breeding system and dependence on pollinators

For testing the mating system we conducted artificial pollination for four treatments: geitonogamy (pollen of the same plant), xenogamy (pollen from other plant), apomixes as asexual reproduction (isolated female flowers) and open pollination (natural), all in previously bagged female flowers for pollen control (except natural pollination). Geitonogamy was only performed when male and female flowers from the same plant were open on the same day. For the xenogamy, we collected pollen and mixed from random individuals at a minimum distance of 10m. We selected female flowers that opened in the day of the treatments. The experiment period was from December 2017 to January 2018. We collected the treatments that developed fruit for seeds count and for analysis of the seed quality through the weight of the fresh seeds. An index of self-incompatibility (ISI) was obtained by dividing the mean number seed set per flower from the treatments of geitonogamy by those from the xenogamy: self-compatible species score 1, incompletely compatible species values between 1 and 0, and self-incompatible species score below 0.2 (Zapata and Arroyo 1978).

Floral morphometrics and nectar traits

For floral measurements, we obtained 32 flowers for each floral type, of different individuals. The collected fresh flowers were photographed using a scale, in three different angles (frontal, lateral and lateral exposing reproductive structures). We used software *ImageJ* to analyze the images. The measurements were linear, consisted of perianth diameter (opposite distance from tip to tip of tepals), perianth length, tepals junction (floral tube), tepal width, flower bottom width, floral nectary diameter and height, and floral pedicel.

Considering that nectar traits are quite responsive to environmental variation and the genetic heritable variation can be lost under field conditions (Mitchell 2004), we sampled nectar in the two first hour of anthesis to diminish environment interference. Each flower had nectar sampling made once, from different individuals in previously bagged flowers, totalizing 10 flowers per sex. The nectar volume was measured with 5 µL micro capillaries for *M. violacea* and 1 µL micro capillaries for *M. oligantha* and the sugar nectar concentration by using a hand refractometer (Atago 0–90% Brix).

Floral visitors, pollinators and functional groups

We performed observation of floral visitors and the entomological collection alternating each activity for 30 minutes, for two consecutive days during the flowering period. Each species had a total of 60h of sampling: in *M. violacea* was 40h for Dom Bosco population in November 2017 to February 2018, and 20h for Urubu population in January 2018; for *M. oligantha* we divided observation into two flowering periods, 40h from November 2017 to February 2018, and 20h from December 2018 to January 2019. The observation hour was equal for FF and MF. The data from the visits consisted of morphospecies, visiting time, floral type, presence of pollen from previous visits, contacts with reproductive floral parts, behavior and accessed floral resource. We collected specimens directly on flowers and analyzed under stereoscopic for taxon determination and observation of pollen on the insect body. The pollen of *Manihot* is easy to be recognized due to the very large size, spherical shape and the exine featuring pila ornaments (see Vieira *et al*. 2012). Specimens are deposited in the Entomological Collection of the Universidade de Brasília (DZUB) (**Appendix**).

We treated as legitimate visits when floral visitors touched the reproductive verticils and carried pollen; the potential pollinators were the insects that visited legitimately and besides it, visited male and female flowers. Further, we grouped the floral visitors and pollinators into functional groups (FG), defined basically as visitors that interacted similarly with the flowers (Fenster *et al.* 2004, Gómez 2009). For FG establishment, we applied criteria of foraging behavior, feeding habits, proboscis length, and body size. We grouped floral visitors into 11 functional groups: ants, bees, flies, grasshoppers, large bees, large beetles, microlepidoptera, short-tongued bees, small bees, small beetles and wasps.

Functional groups importance and specialization of the pollination system

For the estimation of importance in each FG of pollinator, we followed the equation proposed by Armbruster (2014): pollinator importance (PI) = V ×A×S, in which V is the visitation rate per unit time, A is the per-visit probability of contacting the anthers and S is the per-visit probability of contacting the stigmas.

We based on Fenster *et al.* (2004) for the analysis of the specialization-generalization degree that FG that representing three fourths of floral visits frequency can be considered specialized in this FG. Although, these authors discussed that if two FG likely exerts similar selection pressure, specialization can be considered. In contrast, if two FG of pollinators are clearly selecting to different traits due to their habitat, e.g. bats and hummingbirds, this plant species is rather a generalist (Martén-Rodríguez *et al.* 2009).

Statistical analysis

The floral traits related to measurements and nectar (volume and sugar concentration) had a comparison between sexes in each species by one-way ANOVA, through the packages ‘car’ and ‘carData’ (Fox and Weisberg 2011, 2018). Likewise, to test if there were differences among pollination treatments, the seed weight means per fruit followed the mentioned test above. The seed set per fruit were compared between pollination treatments that followed a GLM model with Poisson distribution, by using the package ‘emmeans’ (Lenth 2018). To compare the number of visits to each floral type, we used the chi-square test of goodness-of-fit, with the floral display means ratio as the given probabilities. We created the graphs by using the package ‘ggplots2’ (Wickham 2016). All the statistical analysis was run in R environment v. 3.5.1 (R Core Team 2018).

RESULTS

Anthesis and floral display sex ratio

Floral anthesis was similar between sexes and species, wherein flowers lasted one day. The anthesis was throughout daylight, longer in *M. oligantha* since the flowers opened between 6-7 am and were senescent by 6 pm, while *M. violacea* flowers opened between 11-12 am and also were senescent by 6 pm. The bud in pre-anthesis releases nectar that accumulates inside flowers, on the perianth basis, and if this floral bud was positioned against natural or artificial light, it reflected nectar drops. The male bud had already exposed the pollen when the nectar accumulated. The first movements of perianth are rapid, with centrifugal curling of the tepals. However, the returning movements are slow and in male flowers are characterized when the anthers contract inwards. In *M. oligantha* the perianth started to close midday, with contraction of the anthers at 2 pm. Similar, *M. violacea* contracted anthers at 3 pm.

The flowering occurred during the rainy season of Cerrado (from November to February) for the studied populations. The two sampled populations had male-biased floral display: in *M. violacea* the number of males to female flowers per day was always higher, and had sex ratio of 25 male flowers to 5 female flowers on average; *Manihot oligantha* had a less male-biased floral display, with sex ratio means of 25 male flowers to 18 female flowers. Generally, the number of flowers displayed per individuals was low, and occasionally the sexes overlapped in the same individual (asymmetric protogyny).

Breeding system and dependence on pollinators

The species showed to be dependent on pollen transference to produce seed, thus there was no seed in apomixes treatments (**Table 1**). The seed set per fruit was similar between treatments and the cross and open pollination treatments were not statically different, therefore pollen limitation was not detected for the plant populations. The treatment geitonogamy had lesser seed set per fruit but not statically different from the others. Seeds of geitonogamy had less weight than seeds of natural

Table 1 Seed set and seed weight means ± standard deviation across treatments and sample sizes in parentheses

Species	Seed set per fruit				Seed weight (mg)			Index
	Open	Cross	Self	Apomixis	Open	Cross	Self	ISI
M. violacea	2.20 ± 0.77 (n=33)	2.65 ± 0.48 (n=38)	2.15 ± 0.67 (n=32)	0 (n=39)	60.50 ± 14.38 (n=53)	64.20 ± 16.62 (n=69)	61.70 ± 14.96 (n=43)	0.81
M. oligantha	2.38 ± 0.76 (n=56)	2.86 ± 0.51 (n=35)	2.11 ± 0.78 (n=27)	0 (n=46)	**34.20^{a}** ± 5.56 (n=31)	31.11^{ab}± 3.86 (n=43)	**27.50^{b}** ± 5.97 (n=19)	0.74

Only treatments that show significant difference present superscript letters (in bold, at Tukey's honestly significant difference, t=-2.884, P=0.01).

pollination in *M. oligantha*, revealing that the seeds quality of this former treatment were the lowest. Both species were incompletely compatible according to ISI index.

Dimorphism in floral morphometrics and in nectar traits

The flowers were small sized, and the species had bigger MF concerning floral length and tepal width, and in *M. oligantha,* MF was also larger in perianth width (**Table 2**). The campanulate MF had a short tube, whereas the polytepalous FF many times opened partly, resulting in a shorter tube that functioned as campanulate-shaped. Consequently, perianth diameter size (tepals tip to tip) tent to not differ between the sexes, though FF of *M. violacea* presents a weak tendency to be bigger in diameter. Floral pedicel of FF was wider in both species. The nectary of the species differed between sexes: *M. oligantha* had MF with wider nectary diameter, in contrast, in this species FF had a taller nectary; in *M. violacea* there was only a tendency of nectary in FF to be taller (**Table 2**).

The nectar traits had differences between sexes and were particular to each species (**Figure 2**). *M. oligantha* presented a markedly sexual dimorphism for nectar, with higher volume and concentration in FF. For *M. violacea,* the nectar volume was alike between MF and FF, but sugar concentration had a tendency to be higher in FF.

Table 2 Floral measurements and comparison between floral types showing means ± standard deviation

Morphometric traits	*M. violacea*			*M. oligantha*		
	Female	Male	*F*	Female	Male	*F*
Perianth diameter	17.64 ± 4.47	15.88 ± 3.37	3.22 .	11.23 ± 1.74	11.35 ± 1.78	0.07
Perianth lenght	10.82 ± 1.37	13.12 ± 1.54	39.68***	6.87 ± 0.73	8.61 ± 1.16	51.7***
Perianth tube	3.70 ± 1.63	8.62 ± 1.56	151.7***	2.39 ± 0.88	5.52 ± 0.97	96.66***
Perianth width	6.16 ± 0.93	5.90 ± 0.97	1.19	5.30 ± 0.54	6.24 ± 0.78	31.09***
Tepal width	5.04 ± 0.68	5.61 ± 0.86	8.31**	3.69 ± 0.35	4.31 ± 0.42	40.12***
Nectary diameter	4.56 ± 0.48	4.66 ± 0.67	0.52	4.06 ± 0.24	4.55 ± 0.42	30.72***
Nectary height	1.57 ± 0.20	1.47 ± 0.19	3.87 .	1.15 ± 0.08	1.10 ± 0.09	5.40*
Pedicel	2.74 ± 0.55	1.37 ± 0.29	156.7***	1.31 ± 0.16	1.18 ± 0.15	35.01***

F test significance, 0.07>. P>0.05 marginal significance * P>0.01, ** P>0.001, *** P>0.0001 (n=32 for floral type).

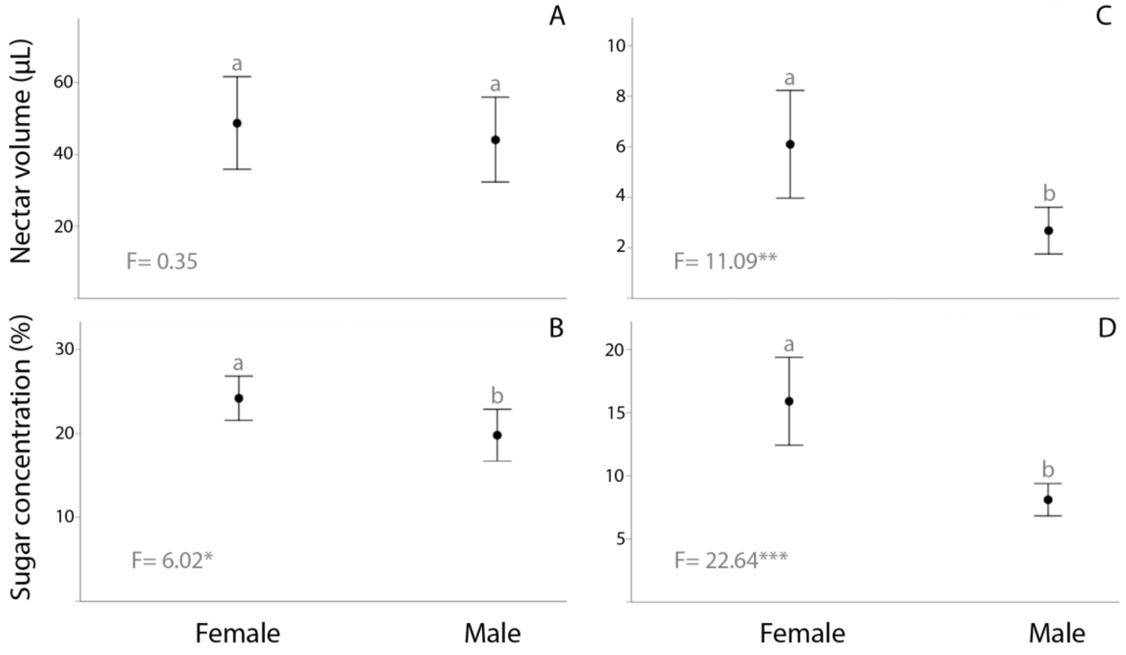

Figure 2 Nectar traits of *M. violacea* in A-B, and *M. oligantha,* C-D. Different letters are significantly different at P<0.05 by F-test.

Generalization of the pollination system and temporal specialization

Practically all the 11 analyzed FG of insect visitors touched anthers and carried pollen, also opportunistic insects contact stigma surface, therefore being legitimate visitors (**Table 3**).

Table 3 Functional groups recorded on *Manihot* flowers

Plant species	Functional group	Body length	Order	Families and genera	Floral resources	Behavioural notes	Type of visit
M. violacea	ants	variable	Hymenoptera	Formicidae (*Camponotus, Dorymyrmex, Pseudomyrmex*)	nectar	long visits; have a protective behavior on female flowers that appears to defend the nectar	legitimate + illegitimate
	bees	variable	Hymenoptera	Apidae (*Ceratina, Partamona, Trigona*)	nectar + pollen	nectaring between tepals, but also hangs on stigma to nectaring; collects pollen	legitimate + illegitimate
	flies	variable	Diptera	Muscidae		land on stigma	legitimate
	grasshoppers	variable	Orthoptera		nectar	nymphs	legitimate
	large beetles	> 7 mm	Coleoptera	Curculionidae	nectar	introduces part of the head in the flowers	legitimate
	large bees	> 10 mm	Hymenoptera	Apidae (*Bombus*)	pollen	entering with ventral part of body in the flower; rapid visit	legitimate
	short-tongued bees	variable	Hymenoptera	Halictidae (*Augochlora, Augochloropsis, Paraxystoglossa, Rhinocorynura*)	nectar + pollen	pollen collecting; explored and gathering on stigma	legitimate + illegitimate
	small beetles	< 3 mm	Coleoptera	Chrysomelidae	nectar	long visits in which stay on the flower bottom robbing nectar; copulated in flowers	legitimate + illegitimate
	small bees	< 3 mm	Hymenoptera	Apidae (*Plebeia*)	nectar	explored the flower without carrying pollen	illegitimate
	microlepidoptera	variable	Lepidoptera		nectar	exploing the nectar around tepals	legitimate
	wasps	variable	Hymenoptera	Vespidae (*Polybia*)	nectar	uses stigma as land surface and hangs on it for nectaring	legitimate + illegitimate

Table 3 (Continued)

Plant species	Functional Group	Body length	Order	Families and genera	Floral resources	Behavioural notes	Type of visit
M. oligantha	ants	variable	Hymenoptera	Formicidae (*Camponotus, Dorymyrmex, Ectatomma, Pseudomyrmex, Solenopsis*)	nectar	long visits; constantly leaves the flowers and returns; agressive with other visitors	legitimate + illegitimate
	flies	variable	Diptera	Chloropidae (*Liohippelates*), Empididae (*Empis*), Lonchaeidae, Muscidae (*Musca, Sarcopromusca*)	nectar + pollen	land on stigma and attracted to the stigma surface; in male fowers can consume pollen	legitimate + illegitimate
	grasshoppers	variable	Orthoptera		nectar + pollen	nymphs	legitimate
	short-tongued bees	variable	Hymenoptera	Halictidae (*Dialictus*)	nectar + pollen	nectaring on tepals, but touches stigma sometimes; collects pollen	legitimate + illegitimate
	small beetles	< 3 mm	Coleoptera	Megalopodidae	nectar	long visits; consume pollen	legitimate
	wasps	variable	Hymenoptera	Vespidae (*Polybia*)	nectar	uses stigma as land surface and hangs on it for nectaring	legitimate

Hymenopterans that formed FG of ants, short-tongued bees and wasps, occurred in both *Manihot* species and the flies FG. The two *Manihot* species had in common three genera of floral visitors: the social wasp *Polybia* and the ants *Pseudomyrmex* and *Camponotus* (**Table 3**). Overall, the floral visitors and pollinators were generalist insects (**Figure 3**).

We found differences among FG in *M. violacea* populations and between the two years of study in *M. oligantha* (**Figure 4**). In *M. oligantha*, FG of ants and flies represented a maximum of 72% and 68% in a flowering, thus the population appeared not specialized in either FG. In *M. violacea*, FG of bees represented 74.50% on average of total visits and both studied populations had

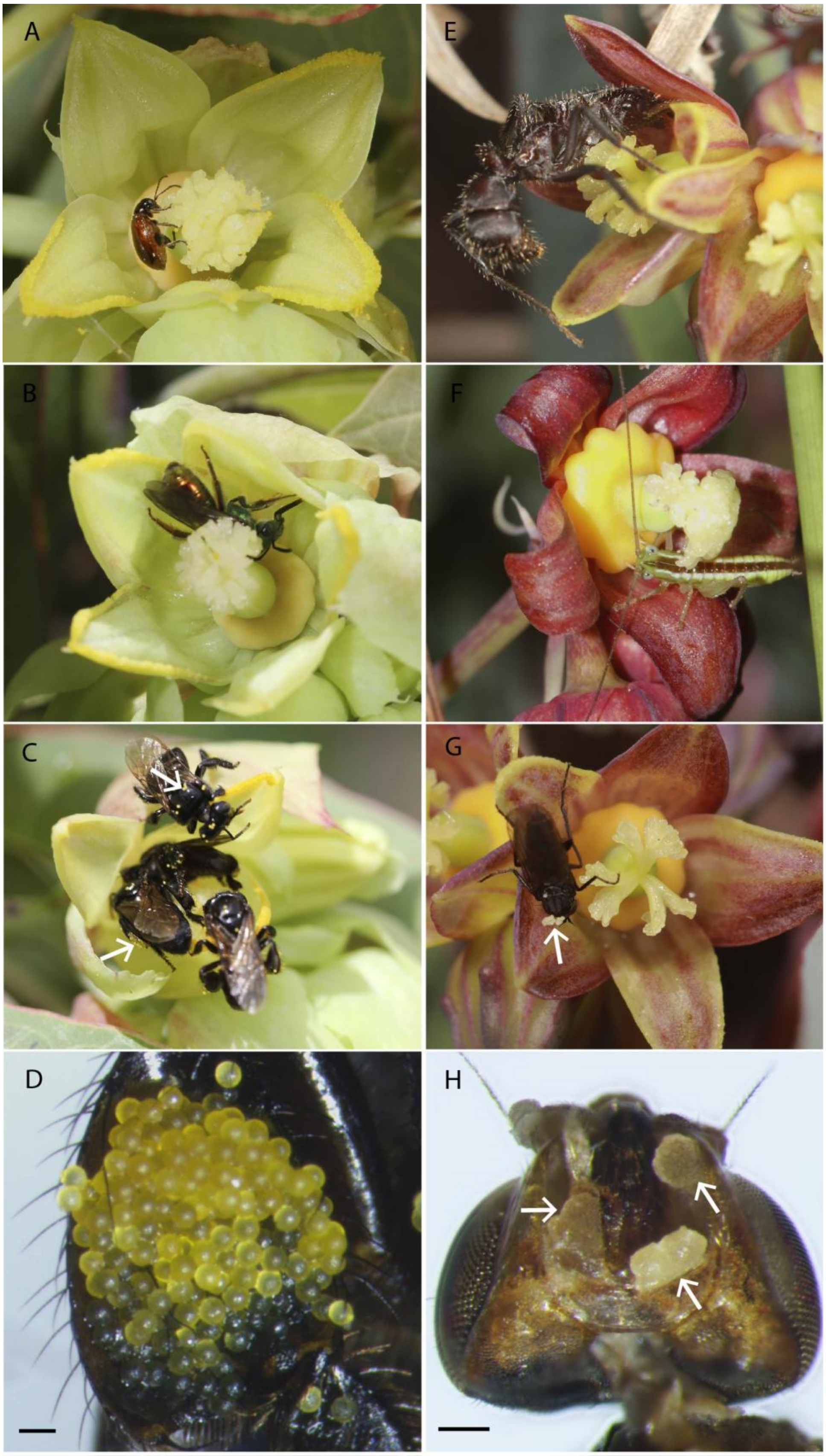

Figure 3 Different functional groups of pollinators on *Manihot* flowers, *M. violacea* A-D, and *M. oligantha* E-H. A, small beetle (Chrysomelidae); B, short-tongued bee (*Augochloropsis*); C, bees (*Partamona cupira*); D, detail of *P. cupira* cobiculae with *M. violacea* pollen; E, ants (*Ectatomma brunneum*); F, grasshopper (nymph); G, fly (*Empis*) carrying pollen (arrow) from *M. oligantha*; H, fly (*Liohippelates*) detail with *M. oligantha* pollen (arrow). Scale bars 0.1 mm.

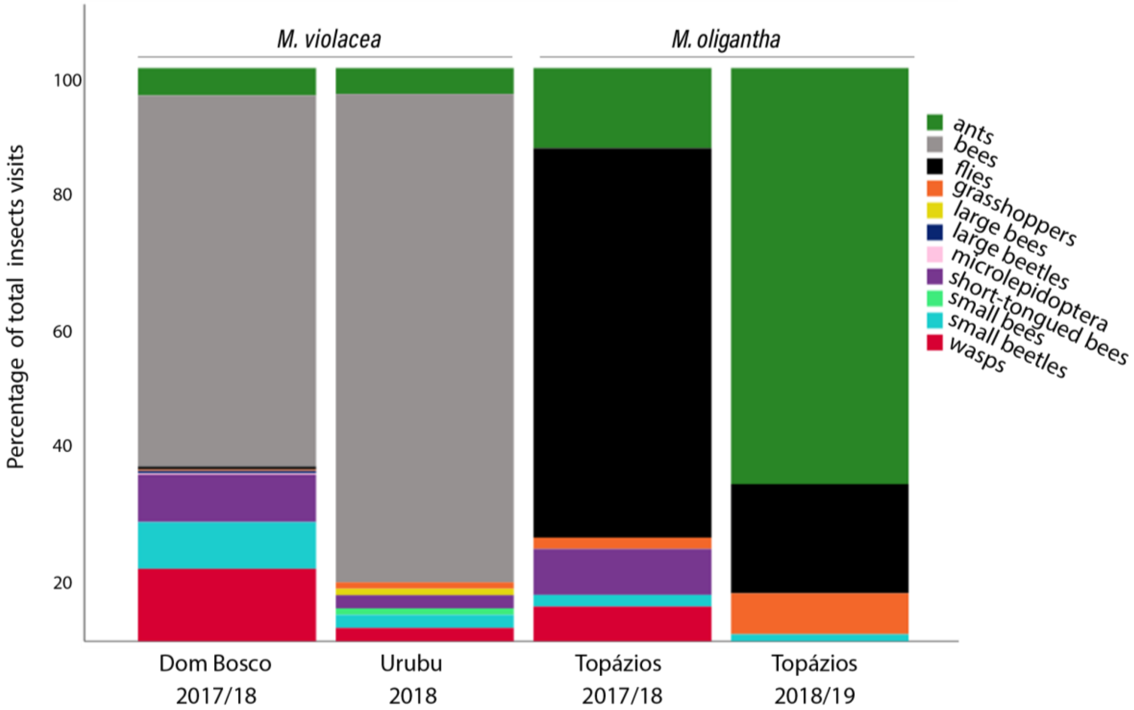

Figure 4 Functional groups of floral visitors for the *Manihot* species, across floral seasons (years 2017-2019) in the study population. Data are expressed as the percentage of total visits for a given flowering season in each population.

FG of bees as the most representative: 94.20% and 66.26% of total visits. Furthermore, 90% of total FG of bees was from a single stingless bee species, the *Partamona cupira*. This bee species could locate the rewarding flower prior to its opening and searched mainly for nectar and less frequently collected pollen (10% of total visits). *Partamona cupira* was the only bee species that during floral anthesis appeared exclusively carrying *M. violacea* pollen (**Figure 3**). The constancy of this bee in *M. violacea* was noteworthy in both sampled populations. As a result, an ecological short-term specialization between the social native bee *P. cupira* and *M. violacea* can be considered at least on the 'Urubu' population.

The pollination system had a high level of generalization, as we found six and five functional groups (FG) of potential pollinators for *M. violacea* and *M. oligantha*, respectively (**Figure 5**). FG of pollinators that appeared the most important were the social bees and wasps for *M. violacea* and the ants and flies for *M. oligantha*. Although, FG that we have seen transferring pollen to stigma were:

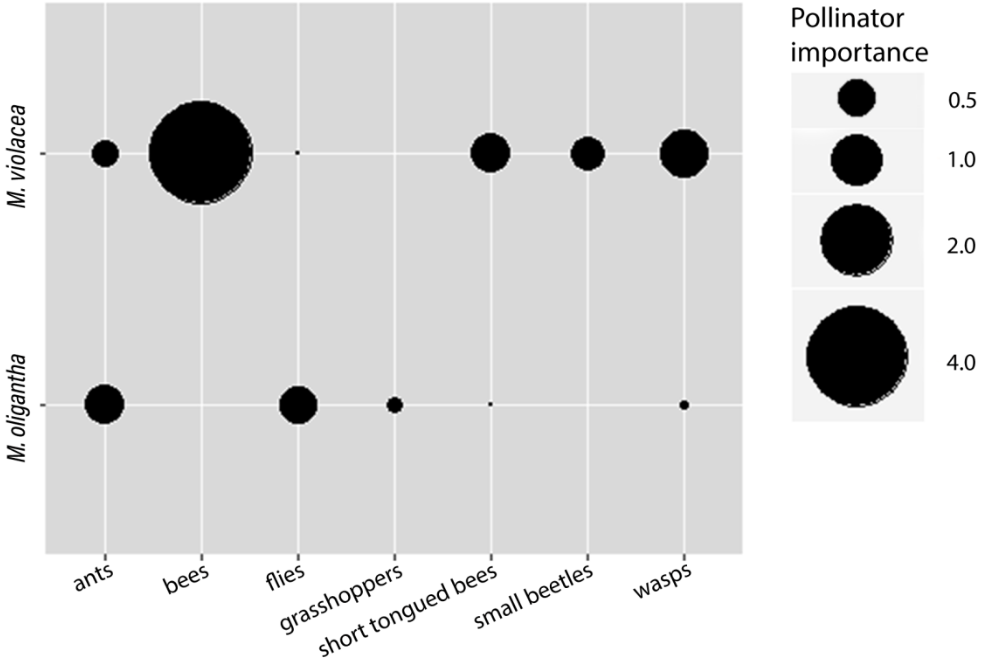

Figure 5 Functional groups of potential pollinators showing their pollinator importance (PI) to each *Manihot* species, combining the sampled sites and flowering seasons.

the bees (n=39), wasps (n=9) and short-tongued bees (n=1) in *M. violacea*, and the flies (n=4) and wasps (n=2) in *M. oligantha*.

Floral visitors and pollinator filtering

Observations revealed that six FG of floral visitors only visited male flowers (**Figure 6**). The relative abundance of floral visitors shown to be a filter in *M. violacea*, in which visitors with less than 0.10 of abundance did not visit female flowers, with the exception of the flies. In *M. oligantha*, the majority of FG visited male and female flowers, though the small beetles FG, which had the lowest abundance did not visit female flowers among the two years. Despite that, several FG of pollinators, including the most important, showed a higher frequency in female flowers than expected (**Figure 7)**, thus this floral type was not dicriminate.

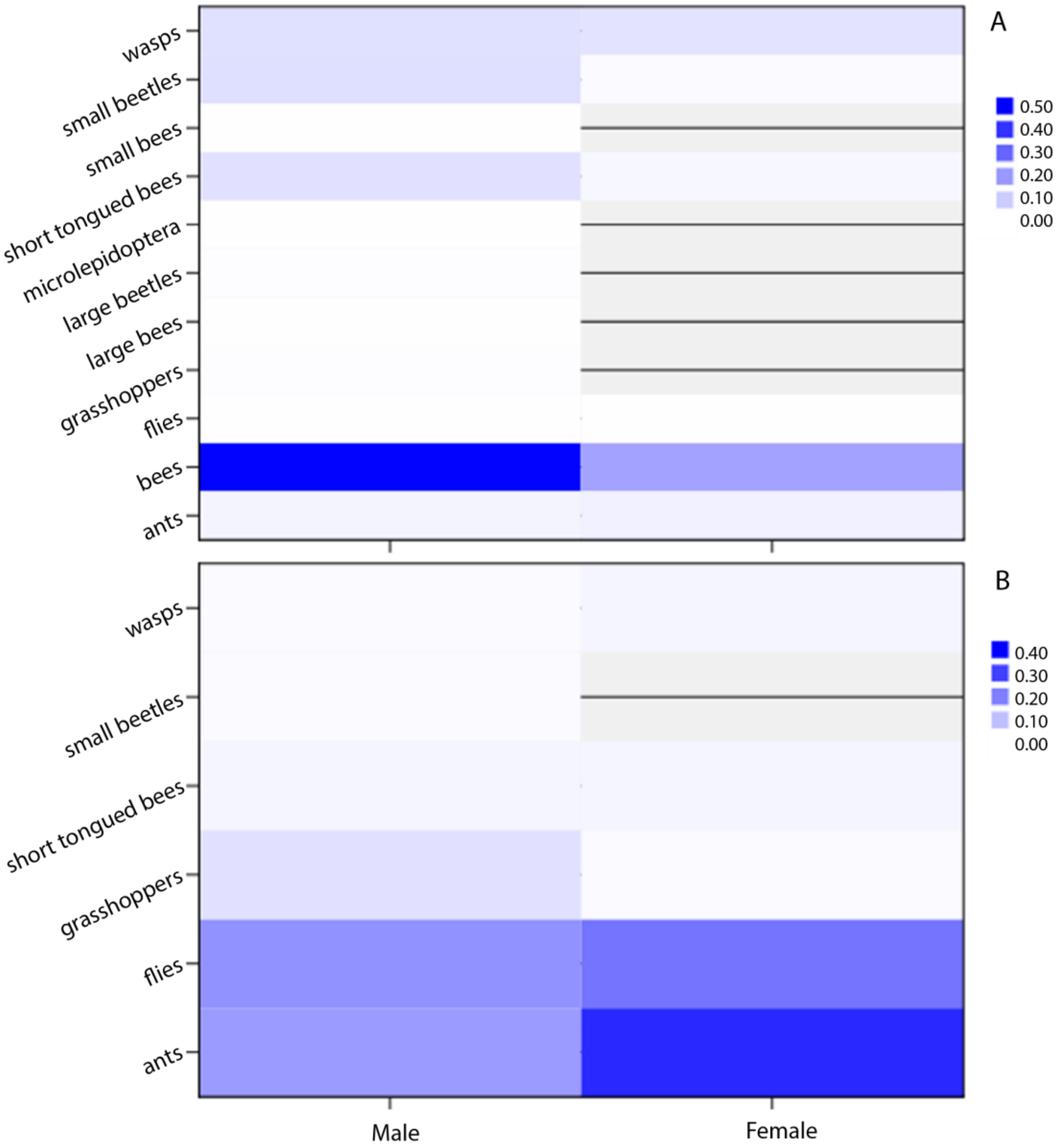

Figure 6 Relative abundance of functional groups among male and female flowers in *M. violacea* A, and *M. oligantha* B. Functional groups that do not visit one floral type are represented in grey with a black line.

We registered *Apis mellifera* in field excursions, in all study areas, however it did not visit *Manihot* flowers. Likewise, stingless bees and solitary bees in the region of *M. oligantha* were identified, though we observed only a short-tongued bee (*Dialictus* sp.) visiting the flowers, in later morning (ca. 11:00). Altogether, the findings indicate an attractant filter of the local fauna (*sensu* Armbruster 2017).

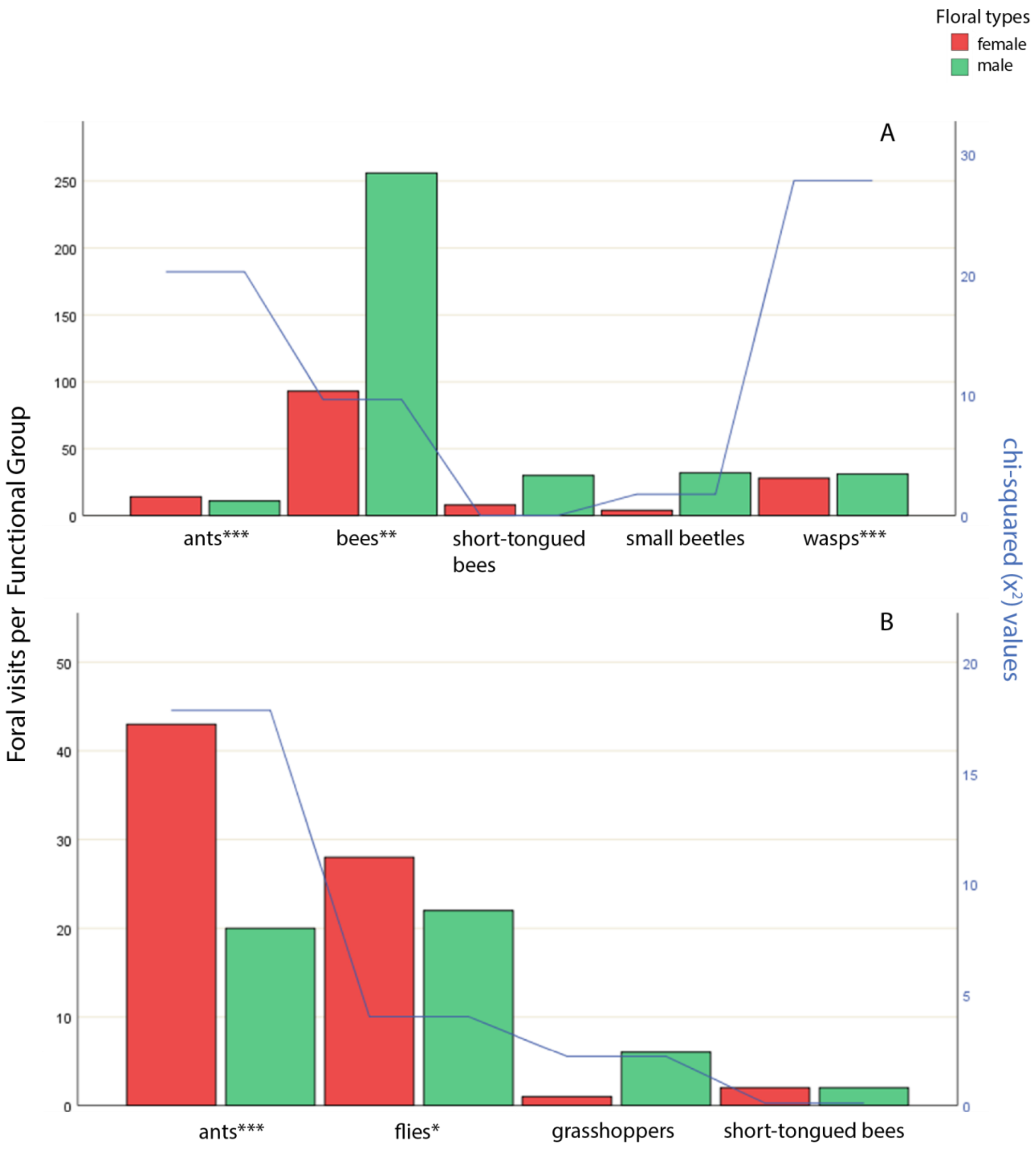

Figure 7 Total visits of functional groups to male and female flowers and the corresponded chi-squared value, in *M. violacea* A, and *M. oligantha* B. Asterisks indicate statistical difference of X^2 values among floral types, for each functional group (* P>0.01, ** P>0.001, *** P>0.0001). Only functional groups of pollinators with five or more visits were included.

DISCUSSION

Generalization and specialization in Manihot species

Considering the number of floral visitors and functional groups that potentially pollinates the species, the system tends to be ecological and functionally generalized. Overall, these pollinators are extremely generalist, varying from social bees and wasps to flies, ants, and grasshoppers. The stamens are arranged in a way that all floral visitors touched it and carried pollen, with the exception of very small ants and bees. Even opportunistic insect groups that appeared with very low relative abundance (e.g grasshoppers), touched stigma. Generalization is advantageous when pollinator assemblage is variable, leading to reproduction assurance (Perfectti *et al.* 2009, Martén-Rodríguez *et al.* 2010) and even though the species show dependence on animal pollination, they are not pollen limited. Considering the heterogeneity of Cerrado environment (Silva *et al.* 2006, Dodonov *et al.* 2014), the generalist systems could be an advantage. The exclusive presence of three ovules in *Manihot* likely contributes to the reproduction assurance and pollination by unspecialized and insects. Furthermore, generalized plants bearing a few ovules did not show difference among their visitors effectiveness-quality component (Gómez and Zamora 1999, Zych *et al.* 2018). In fact, the quantitative component can have a central role in plant reproduction rather than the qualitative component (Vázquez *et al.* 2005, Sahli and Conner 2006) and the most abundant is often the most important pollinators.

Although the pollination system is generalized, the association of *Manihot* with social hymenopterans was strong as both species had bees and ants as the most important pollinators. Therefore, the importance of these social insects for the *Manihot* can be prominent, likely due to their abundance. Indeed, an ecological specialization of short-term could be established between a native stingless bee and *M. violacea* in at least one population. The eusocial bees are known to be related to dioecious and monoecious plants, in which they forage constantly at a specific plant for a while (Bawa 1977, 1980). The *Partamona* bee has high floral fidelity to *M. violacea* that might be related to its unusual foraging strategy of mass-recruiting (Flaig *et al.* 2016). The behavior of *Partamona* can lead to dominance over resources, mainly by the size of a colony that excludes other species (Hrncir and Maia-Silva 2013), in this case, the nectar resource of both unisexual flowers that should be highly attractive for them.

Ants were the most abundant visitors in *M. oligantha*. This species has many traits of plants pollinated by ants, covering nine out ten traits that Hickman (1974) predicts: low height, dense populations (see Simon *et al.* 2018) with intermixed individuals that often grow in places poorly vegetated, small flowers with accessible nectar, between others. The ant pollination is still seen as rare, despite there is crescent evidence of its occurrence and importance (de Vega *et al.* 2009, de Vega and Gómez 2014, Ibarra-Isassi and Sendoya 2016, Domingos-Melo *et al.* 2017; for a different perspective see Rostás *et al.* 2018). Recently, the ant syndrome was unveiled in a Cerrado species - the studied species possessed similar habitat of *M. oligantha*, and the ant agents were shown to be the most effective pollinators (Del-Claro et al 2019). In addition to this qualitative trait, pollination by ants has the quantity component as a key factor, in which the system relies on the abundance of these insects (Gómez and Zamora 1992). However, ant pollination syndrome is related to dry habitats (Hickman 1974, Wyatt 1981) the habitat of *M. oligantha* presented an abundance of water. The anthesis of *M. oligantha* species starts in cold early morning and raining days during blooming were common, under these conditions, instead of ants, flies are the main visitors, and when the weather was hotter the ants visit the flowers (Farinasso, personal observations). The importance of flies in ‘adverse’ conditions was already noticed by Faegri and van der Pijl (1979).

From one season to another, the relative frequency of flies to ants switched in *M. oligantha*. Traits related to fly pollination (myophily) do not possess phenotypic specialization (*sensu* Ollerton *et al.* 2007) that might difficult this syndrome characterization, such as small radial flowers, small nectar quantity and variable concentration, diurnal anthesis and easy access to nectar (Faegri and van der Pijl 1979, Willmer 2011), and all these traits were present in *M. oligantha*. Normally, myophily flowers are visited by other insects orders (Endress 1994), but the flies can represent most frequency and act as main pollinators (Pombal and Morellato 1995, Niemirski and Zych 2011, Zych *et al.* 2013). Generalist plants can adapt to several pollinators groups, and the highest relative frequency of each group is related to its selection power on floral traits (Sahli and Conner 2011). Even if two groups of pollinators present very different effectiveness, the plant is a generalist and can be adapted to these available pollinators (Aigner 2001). It seems that *M. oligantha* featured traits that are suitable for two different and unspecialized groups of pollinators, the ants and flies.

It is assumed that generalist systems are unlikely to fit in pollination syndromes or specialization (Herrera 1989, Gómez and Zamora 1999, Thompson and Wilson 2008, Gómez *et al.* 2014a). However, some were able to qualify an intermediate floral phenotype, pollinated and adapted to completely different functional groups and, despite that, still is characterized as generalist system (Aigner 2005, Martén-Rodríguez *et al.* 2009, 2010, Wilson *et al.* 2017). It was shown that in generalist plants, abundant pollinators might exert strong selection on floral traits, even the low efficient ones (Gómez *et al.* 2014b). While *M. oligantha* presents a floral phenotype that appeared to be adapted to two main functional groups with similar importance, *M. violacea* despite being pollinated by several smaller groups tends to be ecologically specialized in bees. Therefore, our findings support the pollination systems of these two species of *Manihot* as a moderate generalist.

Pollinator filtering in a generalized monoecious system

We suggested that monoecy can function as a filter in the *Manihot* through female flowers. Both species have functional groups that do not visit female flowers, whereas male flowers are always visited. What seems to determine this filtering is both floral display sex ratio and visitor relative abundance. The floral display of the two species of *Manihot* is male-biased with different degrees. Moreover, female display (and not male) in generalist plants was showed to be under a selection of pollinators (Sahli and Conner 2011). The sex ratio of *M. violacea* is male-biased and many functional groups with relatively low abundance are not recorded in female flowers. Differently, the sex ratio of floral display in *M. oligantha* is more similar. It is known that increasing female sex ratio can promote a differential response on pollinators, making female flowers being more visited, and even more similar sex ratio could promote alike visitation (Yvonne and Wardle

2007). In *M. oligantha*, wasps and short-tongued bees visited both floral types even though their relative abundance was very low and only the lowest abundant functional group formed by small beetles did not visit female flowers. Likely due to the balanced sex ratio display, the filtering is more subtle in this latter species.

Our study indicates that the main pollinator groups do not discriminate against female flowers. Actually, nectar traits revealed that female flowers tend to higher volume or sugar concentration and might explain why pollinators visit this floral type more than expected. For the bees, these results are conflicting as this group frequently discriminates against female flowers. In addition to, other studies points out that flies and wasps prefer male flowers (Bell *et al.* 1984, Ashman 2000). However, the female flower had lower nectar volume in the case of these latter authors. In studies that female flowers had higher nectar volume, this floral type was more attractive to pollinators including bees and flies (Gonzalez *et al.* 1995, Cervantes *et al.* 2017). Although in *Manihot* female flowers are smaller in length and pollinators often prefer larger flowers (Conner and Rush 1996, Kawagoe and Suzuki 2003, Parachnowitsch and Kessler 2010), the diameter is similar between sexes, making the size perhaps equally attractive. If female flowers are more attractive to the most important pollinators, what guarantees that they visit male flowers? The number of female flowers could maintain system stability.

Apis mellifera was shown to visits *M. esculenta* (Kawano 1980, Nassar and Carvalho 1990) and this bee is recorded in our field excursions, however, it does not visit the *Manihot* species. Additionally, the richness of bee species visiting the *Manihot* flowers is low when compared to the Cerrado fauna of the group (Pacheco Filho *et al*. 2015). Unfortunately, we could not analyze the chemical composition of nectar, but considering that *Manihot* has many secondary compounds (reviewed in Blagbrough *et al.* 2010) the taste of nectar is probably toxic at some extent. Some compounds such as alkaloids might repel certain floral visitors (Kessler and Baldwin 2007). Alkaloids levels in leaves could be relatively correlated with alkaloids levels in nectar (Adler *et al.*

2006). It is known that *M. esculenta* have prominent alkaloids contents in its leaves (Ebuehi *et al.* 2006), likely nectar contains alkaloids, which might be higher in the studied wild species and thus explains the fact that many bees did not visit the flowers. Besides, pollen collecting was uncommon, suggesting unpalatability. Consequently, the nectar and pollen traits could also act in filtering.

Furthermore, sugar concentration is especially low in male flowers of *M. oligantha*, below the concentration that is suitable for bees (Roubik *et al.* 1995, Biesmeijer *et al.* 1999, Cnaani *et al.* 2006). Indeed, *M. oligantha* barely received visits from bees. Male flowers of this species also featured low nectar volume that consisted with the lower nectary measurements on this floral type. The nectar volume could be influenced by the predominant pollinators and play a big role in driving visitation (Wolff 2006). Little nectar volume might be unattractive and constraint visits by certain pollinators (Heinrich and Raven 1972, Hickman 1974, Willmer and Stone 2004). In contrast to this species, *M. violacea* present nectar with equal volume among floral types and the bees had the greatest importance on pollination. Floral rewards and attraction structures are usually gender-biased (Wilson *et al.* 1994) and nectar rewarding plants with unisexual flowers present similar frequency of biased-nectar for each sex (Willson and Agren 1989, Delph 1996). Depending on which sex function is limited (pollen donation or receipt), it will be favored, i.e. nectar rewarding taxa that are bee-pollinated, the female is frequently the most rewarding flower, whereas in taxa bird-pollinated had generally male-biased nectar (Carlson and Harms 2006). If female flowers are common, attractive traits tent to be female-biased (Ashman and Diefenderfer 2001). In fact, female flowers are abundant in *M. oligantha* floral display and the nectar trait presents markedly sexual dimorphism. Therefore, we suppose that a more attractive female flower is being selected in *M. oligantha*.

In plants with unisexual flowers, as observed in *M. violacea*, it is expected and common the male-biased floral display, due to the less costly male function per flower compared to the female function (Lloyd and Webb 1977, Delph 1996, Costich and Meagher 2001, Huang *et al.* 2006). However, *M. oligantha* have similar sex ratio in the floral display. The selection on the female component in *M. oligantha* can be seen in other floral traits besides the nectar and floral display: in anthesis. The greater investment of this latter species is seen in floral longevity that counts with 11 hours of duration, in comparison with six hours in *M. violacea*. Long floral anthesis might be related to greater efforts to ensure successful pollination (Primack 1985, Ashman and Schoen 1994, Rathcke 2003). If the floral longevity is longer, the species might restrict less the flowers, which seems the case in *M. oligantha*. In contrast, *M. violacea* anthesis schedule is unusual that nectar is offered in the first hottest hours of the day and is readily explored by *Partamona.* It is known that large bees are easier to overheating than small bees, during foraging in full sunlight (Hrncir and Maia-Silva). We suggest that anthesis is restrictive in *M. violacea* and contribute to ecological specialization in this species.

In summary, the monoecy does act as pollinator filtering through female component. The apparent unspecialized flower morphology and the common nectar rewards can lead us to expect several visitors and pollinators. Indeed, we detect many functional groups of potential pollinators in the *Manihot* species, but there are species-specific filters, such as nectar traits and floral anthesis. Our findings reinforce that even generalist systems have floral features that restricts pollinators assemblage.

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APPENDIX

Floral visitors collected in *Manihot* species of the Cerrado

DZUB number	Plant species	Floral types	Coordinate	Date	Order	Family	Genus	Epithet	Author
060958	*M. oligantha*	male	-16.737399, -47.689693	3/i/2018	Coleoptera	Megalopodidae		*sp.*	Latreille, 1802
060971	*M. oligantha*	female	-16.737399, -47.689693	30/xii/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060963	*M. oligantha*	female	-16.737399, -47.689693	30/xii/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060978	*M. oligantha*	female	-16.737399, -47.689693	30/xii/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060983	*M. oligantha*	female	-16.737399, -47.689693	2/i/2018	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060982	*M. oligantha*	male	-16.737399, -47.689693	15/xii/2018	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060964	*M. oligantha*	male	-16.737399, -47.689693	25/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060965	*M. oligantha*	male	-16.737399, -47.689693	3/i/2018	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060966	*M. oligantha*	male	-16.737399, -47.689693	24/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060967	*M. oligantha*	male	-16.737399, -47.689693	25/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060974	*M. oligantha*	male	-16.737399, -47.689693	24/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060969	*M. oligantha*	male	-16.737399, -47.689693	24/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060973	*M. oligantha*	male	-16.737399, -47.689693	25/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060975	*M. oligantha*	male	-16.737399, -47.689693	25/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060979	*M. oligantha*	male	-16.737399, -47.689693	2/i/2018	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060977	*M. oligantha*	male	-16.737399, -47.689693	2/i/2018	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060976	*M. oligantha*	male	-16.737399, -47.689693	25/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060984	*M. oligantha*	male	-16.737399, -47.689693	2/i/2018	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060981	*M. oligantha*	male	-16.737399, -47.689693	24/xi/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060972	*M. oligantha*	male	-16.737399, -47.689693	31/xii/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060980	*M. oligantha*	male	-16.737399, -47.689693	31/xii/2017	Diptera	Chloropidae	*Liohippelates*	*sp.*	Duda, 1929
060968	*M. oligantha*	male	-16.737399, -47.689693	25/xi/2017	Diptera	Lonchaeidae		*sp.*	Loew, 1861
219796	*M. oligantha*	female	-16.737399, -47.689693	15/xii/2018	Diptera	Muscidae	*Sarcopromusca*	*pruna*	(Shannon & Del Ponte, 1926)
060970	*M. oligantha*	female	-16.737399, -47.689693	2/i/2018	Diptera	Muscidae	*Musca*	*domestica*	Linnaeus, 1758
219797	*M. oligantha*	female	-16.737399, -47.689693	15/xii/2018	Diptera	Empididae	*Empis*	*sp.*	Linnaeus, 1758
219823	*M. oligantha*	female	-16.737399, -47.689693	15/xii/2018	Diptera	Empididae	*Empis*	*sp.*	Linnaeus, 1758
219801	*M. oligantha*	male	-16.737399, -47.689693	15/xii/2018	Diptera	Empididae	*Empis*	*sp.*	Linnaeus, 1758

219824	*M. oligantha*	female	-16.737399, -47.689693	15/xii/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
219799	*M. oligantha*	male	-16.737399, -47.689693	15/xii/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
219800	*M. oligantha*	male	-16.737399, -47.689693	15/xii/2018	Hymenoptera	Formicidae	*Camponotus*	*melanoticus*	Emery, 1894
060954	*M. oligantha*	female	-16.737399, -47.689693	2/i/2018	Hymenoptera	Formicidae	*Dorymyrmex*	*brunneus*	Forel, 1908
060961	*M. oligantha*	male	-16.737399, -47.689693	3/i/2018	Hymenoptera	Formicidae	*Dorymyrmex*	*brunneus*	Forel, 1908
060957	*M. oligantha*	male	-16.737399, -47.689693	31/xii/2017	Hymenoptera	Formicidae	*Dorymyrmex*	*brunneus*	Forel, 1908
060956	*M. oligantha*	male	-16.737399, -47.689693	2/i/2018	Hymenoptera	Formicidae	*Dorymyrmex*	*brunneus*	Forel, 1908
219798	*M. oligantha*	female	-16.737399, -47.689693	15/xii/2018	Hymenoptera	Formicidae	*Ectatomma*	*brunneum*	Smith, F., 1858
219794	*M. oligantha*	female	-16.737399, -47.689693	15/xii/2018	Hymenoptera	Formicidae	*Ectatomma*	*brunneum*	Smith, F., 1858
219795	*M. oligantha*	female	-16.737399, -47.689693	15/xii/2018	Hymenoptera	Formicidae	*Ectatomma*	*brunneum*	Smith, F., 1858
219802	*M. oligantha*	male	-16.737399, -47.689693	15/xii/2018	Hymenoptera	Formicidae	*Pseudomyrmex*	*termitarius*	(Smith, F., 1855)
060955	*M. oligantha*	male	-16.737399, -47.689693	2/i/2018	Hymenoptera	Formicidae	*Solenopsis*	*tridens*	Forel, 1911
060962	*M. oligantha*	male	-16.737399, -47.689693	3/i/2018	Hymenoptera	Halictidae	*Dialictus*	*sp.*	Robertson, 1902
060959	*M. oligantha*	female	-16.737399, -47.689693	3/i/2018	Hymenoptera	Vespidae	*Polybia*	*ignobilis*	(Haliday 1836)
060909	*M. violacea*	female	-15.796089,-47.805440	09/xii/2017	Coleoptera	Carabidae		*sp*	Latreille, 1802
061012	*M. violacea*	female	-15.796089,-47.805440	17/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
061007	*M. violacea*	female	-15.796089,-47.805440	19/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
061011	*M. violacea*	female	-15.796089,-47.805440	19/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060928	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060925	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060875	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060927	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060878	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060926	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060899	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060904	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060902	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060903	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060901	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060900	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060897	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060898	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060905	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060988	*M. violacea*	male	-15.796089,-47.805440	19/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
061006	*M. violacea*	male	-15.796089,-47.805440	20/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
061003	*M. violacea*	male	-15.796089,-47.805440	20/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
061004	*M. violacea*	male	-15.796089,-47.805440	20/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060940	*M. violacea*	male	-15.796089,-	22/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille,

			47.805440						1802
061021	*M. violacea*	male	-15.796089,-47.805440	22/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
061022	*M. violacea*	male	-15.796089,-47.805440	22/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060949	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
060995	*M. violacea*	male	-15.717940, -47.851572	30/i/2018	Coleoptera	Chrysomelidae		*sp. 1*	Latreille, 1802
061016	*M. violacea*	female	-15.796089,-47.805440	19/i/2018	Coleoptera	Chrysomelidae		*sp. 2*	Latreille, 1802
060929	*M. violacea*	male	-15.796089,-47.805440	20/i/2018	Coleoptera	Chrysomelidae		*sp. 2*	Latreille, 1802
060919	*M. violacea*	male	-15.796089,-47.805440	20/i/2018	Coleoptera	Chrysomelidae		*sp. 2*	Latreille, 1802
060856	*M. violacea*	male	-15.796089,-47.805440	10/xii/2017	Coleoptera	Curculionidae		*sp.*	Latreille, 1802
060910	*M. violacea*	male	-15.796089,-47.805440	09/xii/2017	Coleoptera	Curculionidae		*sp.*	Latreille, 1802
060834	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Apidae	*Ceratina*	*sp. 1*	Latreille, 1802
060835	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Apidae	*Ceratina*	*sp. 1*	Latreille, 1802
060837	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Apidae	*Ceratina*	*sp. 1*	Latreille, 1802
060838	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Apidae	*Ceratina*	*sp. 2*	Latreille, 1802
061020	*M. violacea*	male	-15.796089,-47.805440	22/i/2018	Hymenoptera	Apidae	*Ceratina*	*sp. 2*	Latreille, 1802
060985	*M. violacea*	male	-15.796089,-47.805440	24/xi/2016	Hymenoptera	Apidae	*Ceratina*	*sp. 3*	Latreille, 1802
060813	*M. violacea*	male	-15.796089,-47.805440	21/xi/2017	Hymenoptera	Apidae	*Ceratina*	*sp. 3*	Latreille, 1802
060836	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Apidae	*Ceratina*	*sp. 3*	Latreille, 1802
061018	*M. violacea*	male	-15.796089,-47.805440	22/i/2018	Hymenoptera	Apidae	*Ceratina*	*sp. 3*	Latreille, 1802
060944	*M. violacea*	male	-15.796089,-47.805440	28/i/2018	Hymenoptera	Apidae	*Ceratina*	*sp. 3*	Latreille, 1802
060937	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Apidae	*Ceratina*	*sp. 3*	Latreille, 1802
061014	*M. violacea*	female	-15.796089,-47.805440	17/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
061010	*M. violacea*	female	-15.796089,-47.805440	17/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
061013	*M. violacea*	female	-15.796089,-47.805440	17/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
061001	*M. violacea*	female	-15.796089,-47.805440	18/iii/2016	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060999	*M. violacea*	female	-15.796089,-47.805440	26/iii/2016	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
028653	*M. violacea*	female	-15.796089,-47.805440	28/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060986	*M. violacea*	male	-15.796089,-47.805440	24/xi/2016	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060987	*M. violacea*	male	-15.796089,-47.805440	24/xi/2016	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060833	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060809	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060913	*M. violacea*	male	-15.796089,-47.805440	02/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060911	*M. violacea*	male	-15.796089,-47.805440	02/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060912	*M. violacea*	male	-15.796089,-47.805440	02/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060915	*M. violacea*	male	-15.796089,-47.805440	02/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060914	*M. violacea*	male	-15.796089,-47.805440	02/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060918	*M. violacea*	male	-15.796089,-47.805440	10/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060854	*M. violacea*	male	-15.796089,-47.805440	10/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)

060917	*M. violacea*	male	-15.796089,-47.805440	10/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060853	*M. violacea*	male	-15.796089,-47.805440	10/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060916	*M. violacea*	male	-15.796089,-47.805440	10/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
061019	*M. violacea*	male	-15.796089,-47.805440	12/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060862	*M. violacea*	male	-15.796089,-47.805440	12/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060859	*M. violacea*	male	-15.796089,-47.805440	12/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060857	*M. violacea*	male	-15.796089,-47.805440	12/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060858	*M. violacea*	male	-15.796089,-47.805440	12/xii/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060883	*M. violacea*	male	-15.796089,-47.805440	17/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060881	*M. violacea*	male	-15.796089,-47.805440	17/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060880	*M. violacea*	male	-15.796089,-47.805440	17/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060906	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060877	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060840	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060996	*M. violacea*	male	-15.796089,-47.805440	26/iii/2016	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060935	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060933	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060930	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060936	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060932	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060931	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060950	*M. violacea*	male	-15.717940, -47.851572	30/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060943	*M. violacea*	male	-15.717940, -47.851572	30/i/2018	Hymenoptera	Apidae	*Partamona*	*cupira*	(Smith, 1863)
060947	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Apidae	*Plebeia*	*sp.*	Schwarz, 1938
060817	*M. violacea*	male	-15.796089,-47.805440	21/xi/2017	Hymenoptera	Apidae	*Trigona*	*spinipes*	(Fabricius, 1793)
061008	*M. violacea*	female	-15.796089,-47.805440	19/i/2018	Hymenoptera	Formicidae	*Camponotus*	*blandus*	(Smith, F., 1858)
061009	*M. violacea*	female	-15.796089,-47.805440	19/i/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
060994	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
060993	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
060921	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
060922	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
060992	*M. violacea*	male	-15.796089,-47.805440	19/i/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
060991	*M. violacea*	male	-15.796089,-47.805440	19/i/2018	Hymenoptera	Formicidae	*Camponotus*	*crassus*	Mayr, 1862
060946	*M. violacea*	female	-15.717940, -47.851572	30/i/2018	Hymenoptera	Formicidae	*Camponotus*	*novogranadensis*	Mayr, 1870
060942	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Formicidae	*Camponotus*	*novogranadensis*	Mayr, 1870
060941	*M. violacea*	female	-15.717940, -47.851572	30/i/2018	Hymenoptera	Formicidae	*Dorymyrmex*	*sp.*	Mayr, 1866
060876	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Hymenoptera	Formicidae	*Pseudomyrmex*	*termitarius*	(Smith, F., 1855)
060814	*M. violacea*	male	-15.796089,-	21/xi/2017	Hymenoptera	Halictidae	*Augochlora*	*sp. 1*	Smith,

			47.805440						1853
060920	*M. violacea*	male	-15.796089,-47.805440	20/i/2018	Hymenoptera	Halictidae	*Augochlora*	*sp. 2*	Smith, 1853
060832	*M. violacea*	male	-15.796089,-47.805440	09/xii/2017	Hymenoptera	Halictidae	*Augochlora*	*sp. 3*	Smith, 1853
060815	*M. violacea*	female	-15.796089,-47.805440	21/xi/2017	Hymenoptera	Halictidae	*Augochloropsis*	*sp. 1*	Cockerell, 1897
060811	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Halictidae	*Augochloropsis*	*sp. 1*	Cockerell, 1897
060860	*M. violacea*	male	-15.796089,-47.805440	12/xii/2017	Hymenoptera	Halictidae	*Augochloropsis*	*sp. 1*	Cockerell, 1897
060938	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Halictidae	*Augochloropsis*	*sp. 1*	Cockerell, 1897
060884	*M. violacea*	male	-15.796089,-47.805440	17/i/2018	Hymenoptera	Halictidae	*Augochloropsis*	*sp. 2*	Cockerell, 1897
061015	*M. violacea*	male	-15.796089,-47.805440	21/i/2018	Hymenoptera	Halictidae	*Augochloropsis*	*sp. 3*	Cockerell, 1897
060818	*M. violacea*	male	-15.796089,-47.805440	21/xi/2017	Hymenoptera	Halictidae	*Paraxystoglossa*	*sp.*	Moure, 1941
060816	*M. violacea*	male	-15.796089,-47.805440	21/xi/2017	Hymenoptera	Halictidae	*Paraxystoglossa*	*sp.*	Moure, 1941
060810	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Halictidae	*Paraxystoglossa*	*sp.*	Moure, 1941
060839	*M. violacea*	male	-15.796089,-47.805440	22/xi/2017	Hymenoptera	Halictidae	*Paraxystoglossa*	*sp.*	Moure, 1941
060879	*M. violacea*	male	-15.796089,-47.805440	17/i/2018	Hymenoptera	Halictidae	*Rhinocorynura*	*sp.*	Schrottky, 1909
060882	*M. violacea*	male	-15.796089,-47.805440	17/i/2018	Hymenoptera	Vespidae	*Polybia*	*occidentalis*	Olivier, 1791
060812	*M. violacea*	female	-15.796089,-47.805440	21/xi/2017	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
061005	*M. violacea*	female	-15.796089,-47.805440	17/i/2018	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
060831	*M. violacea*	male	-15.796089,-47.805440	09/xii/2017	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
060945	*M. violacea*	male	-15.796089,-47.805440	09/xii/2017	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
060952	*M. violacea*	male	-15.796089,-47.805440	09/xii/2017	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
060855	*M. violacea*	male	-15.796089,-47.805440	10/xii/2017	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
060861	*M. violacea*	male	-15.796089,-47.805440	12/xii/2017	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
061017	*M. violacea*	male	-15.796089,-47.805440	17/i/2018	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
060948	*M. violacea*	male	-15.717940, -47.851572	30/i/2018	Hymenoptera	Vespidae	*Polybia*	*sericea*	(Olivier, 1791)
060939	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Hymenoptera	Vespidae	*Polybia*	*sp.*	Lepeletier, 1836
060990	*M. violacea*	male	-15.796089,-47.805440	19/i/2018	Lepidoptera			*sp.*	Linnaeus, 1758
060923	*M. violacea*	male	-15.796089,-47.805440	18/i/2018	Orthoptera			*sp.*	Latreille, 1793
060934	*M. violacea*	male	-15.717940, -47.851572	29/i/2018	Orthoptera			*sp.*	Latreille, 1793