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PALYNOLOGY AND MEIOTIC BEHAVIOR OF *Genipa americana* L., A SPECIES NATIVE TO THE AMAZON

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Abstract

Genipa americana L., commonly known as genipap, belongs to the Rubiaceae family. This study aimed to describe the pollen morphology of the species, evaluate its meiotic behavior and pollen viability, and provide information to help the maintenance and conservation of the species in its natural habitat. Flower buds were collected from 20 individuals in Alta Floresta and Matupá municipalities, Mato Grosso, Brazil. Pollen morphology was characterized using acetolysis and compared to existing literature. Meiotic and post-meiotic phases were analyzed using 2% acetocarmine stain, and pollen viability was estimated using Sudan IV, Alexander's stain, Lugol's solution (1%), and 2% acetocarmine stain. *G. americana* has medium-sized, 3-colporate pollen with reticulated exine and few meiotic irregularities. Acetocarmine stain showed the highest mean percentage of pollen viability (97.96%). Stain tests revealed significant differences, indicating high pollen viability and meiotic regularity. However, conservation and recovery of degraded areas are still necessary as there is no guarantee of successful reproduction due to factors associated with fragmentation, genetic drift, reduced gene flow, and inbreeding.

Keywords: Colorimetric tests. Genipap tree. Pollen grains.

1. Introduction

The genipap tree (*Genipa americana* L.), a native species of the Rubiaceae family, occurs in all tropical countries of the Americas. In Brazil, it can be found in all biomes and has solid economic potential in the northeastern states because its wood is used in civil construction, crafts, and furniture manufacturing. Its fruit is edible when fresh or in the form of sweets and liqueurs, and all parts of the plant have medicinal properties (Moura et al. 2016; Cardoso et al. 2020; Gomes 2020; Souza and Vieira 2020). The species is functionally dioecious, with pollination made by flies and bees (Andrade Bortoleti et al. 2018). Genipap seeds are dispersed by fish and mammals such as the tapir (*Tapirus terrestris* L.) and the red brocket deer (*Mazama* sp.), as reported by interviewed genipap harvesters (Ruzza et al. 2020).

The conservation of the genipap tree, along with other native species, is a concern due to the risk of habitat loss resulting from human actions. Costa et al. (2019) emphasize the need to consider the frontier effect in territorial planning. Fragmentation of areas increases carbon emissions (Vijay et al. 2016; Zemp et

al. 2017) and restricts the ecological functions of species in general, such as mutualism and interactions between species. In the case of tropical forests, fragmentation has more severe consequences due to the specialized interaction of plants with seed dispersers and pollinators, as well as allogamy reproduction (Matos et al. 2018).

Habitat loss can result in genetic loss, altered distribution patterns, and even extinction if species reproduction is compromised. Therefore, it is necessary to conserve species *ex situ* and *in situ* (Arruda et al. 2018; Spoladore et al. 2017; Moraes et al. 2018; Garcia et al. 2019). Small populations are particularly vulnerable to genetic drift, as their reproductive mechanisms are directly affected. The lower pollen grain availability can increase inbreeding, modify gene flow, and limit genetic diversity (Aguilar et al. 2019).

Pollen morphology varies among species, making it necessary to conduct palynological studies to characterize pollen grains and identify the corresponding species. These studies contribute to various fields such as botany, paleontology (Zhao et al. 2019), melissopalynology (Souza 2018), and forensic investigations (Laurence and Bryant 2019; Alotaibi et al. 2020), among others. For pollen morphology analysis, the cellular contents must be extracted to facilitate the visualization of the sexine and the pollen apertures. For this purpose, the acetolysis method proposed by Erdtman (1952) is used, which involves the use of acetic anhydride and sulfuric acid.

Abnormalities during microspore formation can result in non-viable pollen grains, potentially causing unsuccessful reproduction. Techniques such as pollen tube germination *in vitro* and *in vivo*, as well as colorimetric tests, are employed to assess pollen viability. *In vitro* tests involve preparing a culture medium, while *in vivo* tests evaluate fertilization of manually deposited pollen on flowers. Colorimetric tests are recommended for analyzing cell constitution and integrity using stains (Einhardt et al. 2006) such as Alexander's stain, acetocarmine, Lugol's solution, and Sudan IV. Acetocarmine stain indicates chromosome integrity, Lugol's solution infers the presence of starch, Sudan IV stain suggests the presence of lipids, and Alexander's solution reacts with protoplasm and the cellulose of the pollen wall (Dafni 1992; Munhoz et al. 2008).

Due to the ecological, economic, and medicinal significance of genipap trees and their susceptibility to human impact, this study aims to describe the pollen morphology of *Genipa americana* and evaluate its meiotic behavior and pollen viability. This information will help the maintenance and conservation of the species in its natural habitat.

2. Material and Methods

The collections were performed in the municipalities of Alta Floresta (09° 52' 32" S, 56° 05' 10 "W) and Matupá (10° 03' 27" S, 54° 55' 58" W), located in the north of the Brazilian state of Mato Grosso (Figure 1), during the flowering period, which occurs between August and October in this region. Flower buds were collected from *G. americana* individuals located at the edges of forest fragments and in areas of urban and agricultural use. Flower buds were collected from ten individuals in each municipality.

Collection of plant material

We collected 15 flower buds from each of the 20 functionally male individuals of *G. americana*, which varied in size and development stage, for observation. Flower buds in the pre-anthesis phase were selected for analysis. The buds were fixed in a solution of ethanol and acetic acid (3:1, v/v) for 24 hours and then stored in 70% ethyl alcohol at 4°C until further evaluation of morphology, meiosis, post-meiosis products, and pollen viability.

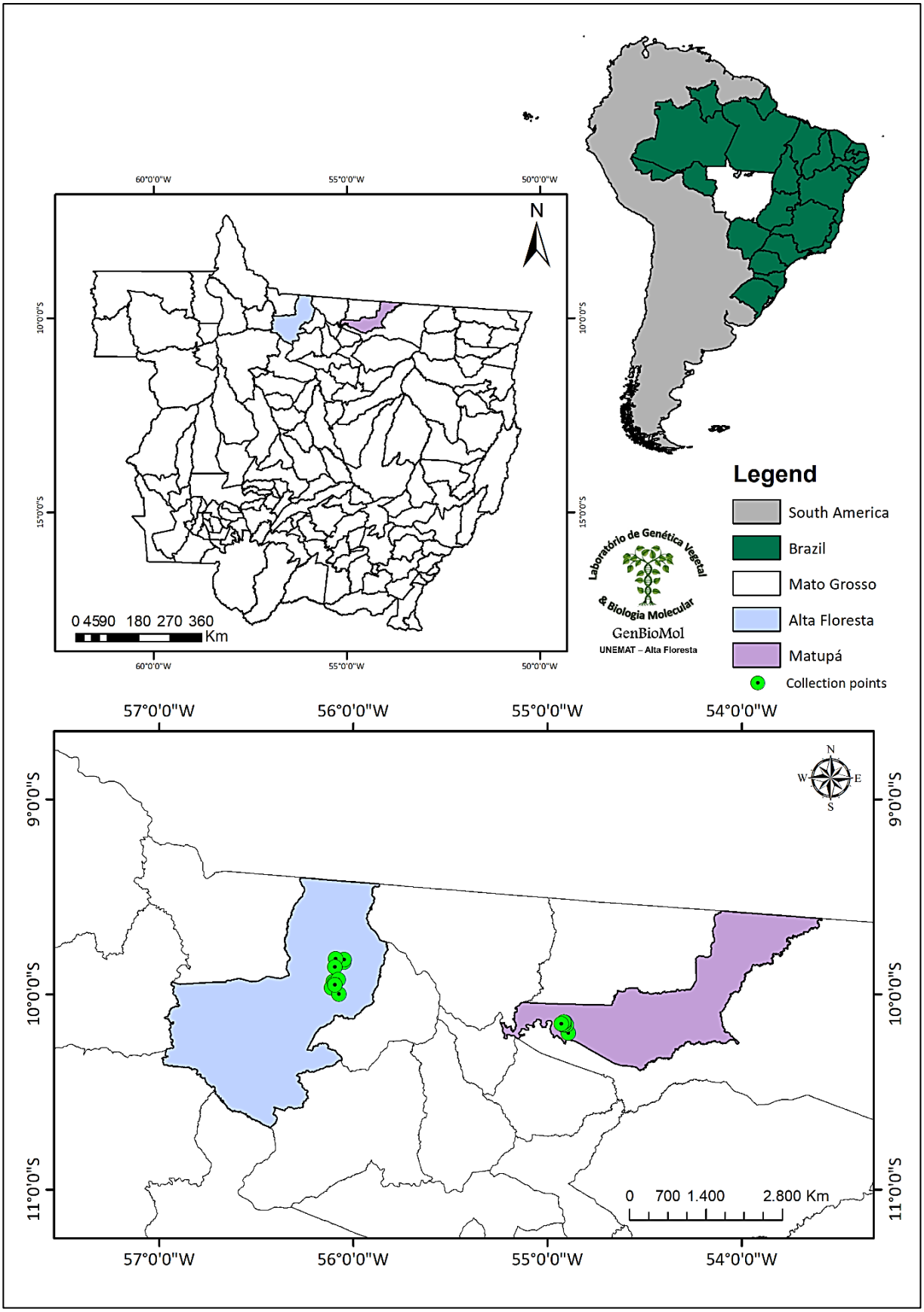

Figure 1. Geographic location of the collection points of the *Genipa americana* samples obtained in the municipalities of Alta Floresta e Matupá, Mato Grosso state, Brazil.

Morphological characterization of pollen

The acetolysis method by Erdtman (1952) analyzed pollen morphology. Measurements were taken from photos of pollen taken on the same day of slide preparations to avoid possible issues with intumescence and changes in pollen size over time.

We measured the polar and equatorial diameters of pollen grains in the equatorial view (pollen grain perpendicular to the polar view) and the equatorial diameter in the polar view (pollen grain with the polar area facing the observer), as well as the thickness of exine layers (sexine and nexine). At least five pollen grains per slide were measured, and 25 measurements of each characteristic were taken, resulting in 25 pollen grains in the equatorial view and 25 in the polar view.

According to Erdtman (1945), pollen grains were classified per size based on the length of the largest axis into the following categories: very small (<10 µ), small (10-25 µ), medium (25-50 µ), large (50-100 µ), very large (100-200 µ), and gigantic (>200 µ). To classify pollen grains based on shape, we used the polar and equatorial axis (P/E) relationship in an equatorial view, as proposed by Erdtman (1952). The polynomial descriptions and terminologies used were based on the glossary of Barth (1965) and Punt et al. (2007). Pollen was classified with the polar area index (PAI) proposed by Barth and Melhem (1988, cited by Martins 2010).

Pollen grain images were captured using a Leica DMLB photomicroscope and analyzed and measured using the Anati Quanti 2® UFV program (Aguiar et al. 2007).

Meiotic behavior

Two anthers per flower bud of each previously fixed individual were macerated on a slide with 2% acetocarmine stain. The material was then observed under an optical microscope, and the different phases of meiosis were analyzed and photographed. Normal stages and meiotic irregularities were also captured.

Post-meiotic phase

For estimating the meiotic index (MI), 1,500 post-meiotic products were counted on six slides, with 250 cells per slide. We considered tetrads with four cells of the same size as normal and any deviation (monad, dyad, triad, and polyad) as abnormal. MI was calculated with the expression proposed by Love (1951), where:

- MI = [(*total number of normal tetrads / total number of monads + dyads + triads + tetrads + polyads*)] x 100.

The recombination index was estimated by analyzing 95 cells in the diakinesis phase, according to Darlington (1958), using the formula:

- RI = ([∑ *total number of chiasmas / number of cells analyzed] + n value*) x 100, where n is the haploid number of the species.

Descriptive statistics were conducted on the data obtained from the evaluations using the GENES program (Cruz 2016).

Pollen viability

Colorimetric tests were performed using four different stains: 2% acetocarmine, 1% Lugol's solution, Sudan IV, and Alexander's stain. To prepare the slides, two anthers per flower bud, previously fixed in Carnoy's solution, were macerated on a slide in drops of each stain. A total of 250 cells were counted per slide, and ten slides were prepared for each stain, resulting in 2,500 pollen grains per stain. The data were analyzed with the GENES program (Cruz 2016), aided by the simple random sampling method and a confidence interval for proportions.

3. Results

Morphological characterization of pollen

The pollen grains of *G. americana* are 3-colporate, with an equatorial diameter ranging from 31.32 to 43.55 µm and a polar diameter from 28.28 to 44.49 µm. They are considered medium-sized when

ranging from 25 to 50 µm. Exine has an average measure of 2.94 µm and is cross-linked with sexine (Figure 2). *G. americana* pollen is classified as oblate-spheroidal, with a small polar area (Table 1).

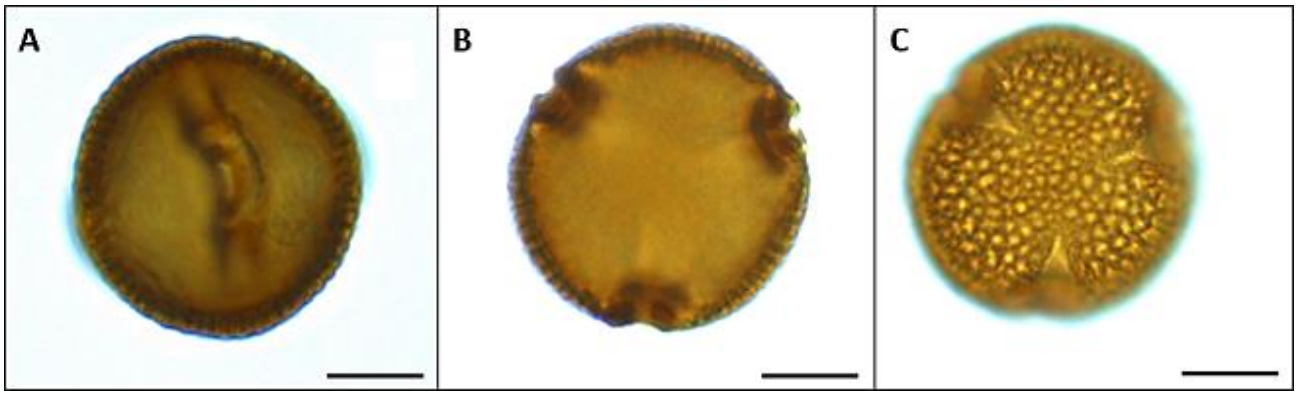

Figure 2. Photomicrographs of the pollen grains of *Genipa americana*. A) polar view showing the colpi; B) equatorial view; C) surface detail. Bar= 10µm.

Table 1. Measurements of the pollen of *Genipa americana* L.

	x ± sx	CI 95%	CV %
Polar diameter (EV)	27.44 - 44.35 µm	37.45 µm	6.11
Equatorial diameter (EV)	31.32 - 43.55 µm	38.51 µm	2.74
Equatorial diameter (PV)	28.28 - 44.49 µm	34.88 µm	3.39
Nexine	1.20 - 2.52 µm	1.65 µm	0.31
Sexine	1.23 - 2.02 µm	1.65 µm	0.23
Exine	2.4 - 3.87 µm	2.94 µm	0.33
P/E	0.97µm		Oblate-spheroidal
P A I	0.36µm		Small polar area

P/E = ratio of polar axis to equatorial diameter; PAI = polar area index; x: median; sx: standard deviation from mean; CI: confidence interval; CV (%): coefficient of variation; EV: equatorial view; PV: polar view.

Meiotic behavior

The meiotic analysis showed 11 pairs of chromosomes or bivalents in the cells during diakinesis (Figure 3). There was also one pair of rod chromosomes, which indicates the occurrence of a chiasma, and ten ring pairs, which suggests two chiasmas in most cells. However, we observed some cells with up to three rod and eight ring chromosomes. The recombination index (RI) for *G. americana* was 19.71% (Table 2).

Table 2. Chiasmas observed in *G. americana* and calculation of the recombination index.

Chiasmas	Total
Ring	648
Rod	179
Total cells	95
IR= (∑ chiasmas⁄total cells) + n	19.71%

Meiosis in the genipap tree was regular, but it showed some abnormalities. The most common ones consisted of chromosomes with irregular segregation, such as premature chromosomes in metaphase I and II and delayed chromosomes in anaphase I and II. There were also cells with cellular asynchrony in meiosis II, in which 30.48% had one of their chromosome groups in anaphase II and telophase II. *G. americana* had 76.92% normal cells (Table 3).

Post-meiotic phase

The analysis of post-meiotic products indicated that the levels of meiotic irregularities in *G. americana* are low, as the MI was 98.80%. There were no dyads or polyads.

Table 3. Percentage of abnormalities observed in meiosis of *G. americana.*

Abnormality	% of abnormality
Premature segregation (metaphase I and II)	10.97%
Delayed chromosome (anaphase I and II)	58.55%
Asynchronous division	30.48%
Number of abnormal cells	82
Total cells analyzed	259
% of abnormal cells	31.66%

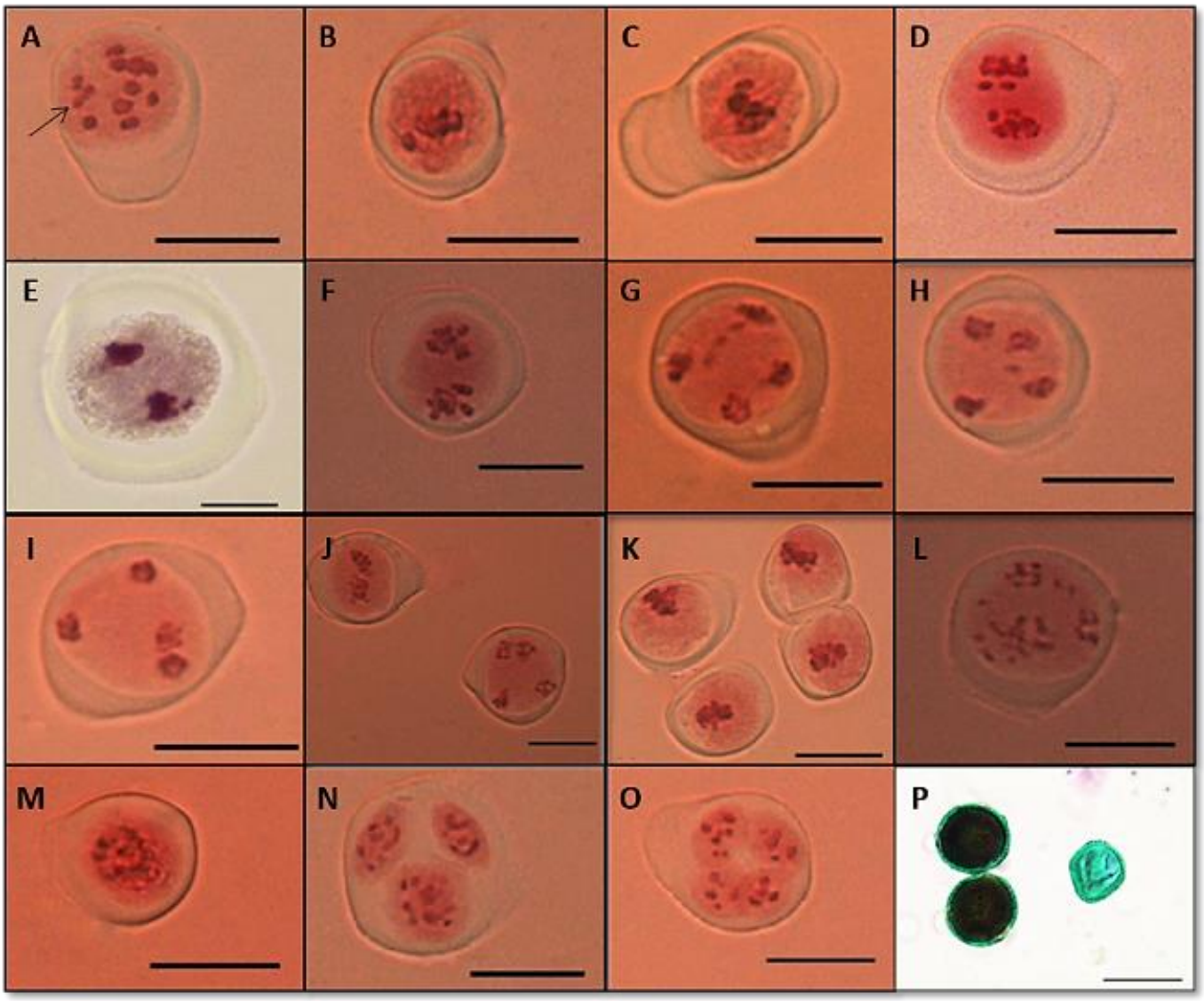

Figure 3. Meiosis in *Genipa americana* L. A - Diakinesis presenting 11 chromosomal pairs; 10 bivalents in ring-type meiotic configuration and one bivalent in rod form (arrow) were observed. B – Metaphase I showing a premature-separating chromosome. C – Metaphase I evidencing the spindle fibers. D – Anaphase I presenting two delayed chromosomes. E – End of telophase I and beginning of metaphase II, showing a chromosome moving away early. F-G – Anaphase II presented delayed chromosomes and lack of synchrony in the cell. H – Anaphase II with a delayed chromosome. I - Anaphase II showing lack of synchrony in the cell. J – Lack of synchrony in the cell division. K – Lack of uniformity of the metaphase equatorial plate. L – Irregular segregation of chromosomes. M – Monad. N – Triad. O – Normal tetrad. P – Pollen grain: viable (dark = purple) and unviable (light = green). Bar (A-D and F-O) = 20 µm; Bar (E) = 10 µm; Bar (P) = 50 µm.

Pollen viability

The Sudan IV stain test showed that 88.04% of the grains were stained, indicating the presence of lipids. The acetocarmine stain revealed intense red staining in 97.96% of the pollen grains, inferring chromatin integrity.

The Alexander's stain test indicated that 81.52% of the grains had an intact protoplasm and cell wall, evidenced by the violet protoplasm coloring and the green cell wall contour. Pollen grains lacking protoplasm and an intact cell wall had a greenish hue. The Lugol's solution test revealed starch in more than 92.56% of the pollen grains, which turned brown with the stain.

According to Table 4, the acetocarmine stain promoted the highest mean viability percentage in *G. americana*, which did not statistically differ from the means obtained with Lugol's solution and the Sudan IV stain.

Table 4. Average percentage of viability of *G. americana* pollen resulting from colorimetric tests.

Stains	% of stained grains
Acetocarmine 2%	97.96 a
Lugol 1%	92.56 ab
Sudan IV	88.04 ab
Alexander's stain	81.52 b

Means followed by the same letter do not differ from each other by the Tukey test ($p \leq 0.05$).

4. Discussion

The genipap tree pollen is similar to that of *Leptodermis purdomii*, *Leptodermis buxifolia*, and *Ixora venulosa* Benth., which all belong to the Rubiaceae family (Dutra et al. 2020; Guo et al. 2020). However, they differ from each other in size and ornamentation. The peculiarities of pollen characteristics allow for the fertilization of the species by the appropriate pollen. According to Moore and Webb (1978), cited by Martins (2010), chemical compounds (released from the disintegration of carpet cells) called "recognition" proteins are stored in the apertures of the pollen ornamentation, and are responsible for pollen germination in the compatible stigma. The genipap tree pollen has a reticulated sexine ornamentation.

The nexine and sexine of *G. americana* have similar measurements, constituting an exine thicker than 2 µm. *G. americana* pollen is medium-sized, oblate-spheroidal, and tricolporate, with a small polar area correlated with the type of long aperture. The characteristics analyzed in this study presented slightly higher values than those by Dutra et al. (2020). This variation may be due to the regional location of the individuals used in the studies; in this work, individuals from the Amazon biome were analyzed, while the cited authors investigated individuals from the Cerrado biome.

The pollen viability of plant species is crucial for successful reproduction, and it depends on meiotic regularity. The observation of 11 pairs of chromosomes in diakinesis confirmed that *G. americana* is diploid (2n=2x=22). Although *G. americana* showed premature chromosome separation, delayed chromosomes, and asynchrony in cell division, its meiotic index was stable and exceeded 90%.

The 1% Lugol's solution and Sudan IV stain indicated the presence of starch and lipids in over 85% of the genipap tree pollen. However, some studies advise against using these tests to estimate pollen viability because they may overestimate the data, as even non-viable pollen can contain these substances (Einhardt et al. 2006; Munhoz et al. 2008).

Several studies recommend colorimetric tests for pollen viability due to the practicality in differentiating between viable and non-viable pollen and the presence of substances that react with the stain, such as Lugol's solution (starch), Sudan IV (lipids), acetocarmine (chromatin integrity), and Alexander's solution (nucleus integrity) (Alexander 1969; Dafni 1992; Martins 2010; Jesus et al. 2018; Santos and Añez 2018; Furini et al. 2020).

Acetocarmine verifies chromatin integrity, and its use showed intact chromatin in 97.96% of *G. americana* pollen. Alexander's stain revealed the lowest viability percentage for *G. americana*, with 81.52% viable pollen, indicating the high pollen viability of the species. This stain helps evaluate aborted or non-aborted pollen, reflecting the integrity of the nucleus and plasma membrane due to its malachite green

and acid fuchsin composition. Besides estimating pollen without a nucleus (non-viable), this stain can also differentiate viable from non-viable pollen (Hister and Tedesco 2016). Munhoz et al. (2008) also endorse this tool for taxonomists in identifying hybrids, as they may not present a nucleus or have altered nuclei.

G. americana demonstrated high pollen viability, exceeding 80% when subjected to colorimetric tests using the four stains, indicating the species' potential for successful reproduction. The presence of meiotic irregularities alongside high pollen viability suggests that the species may have undergone cell repair mechanisms to correct any meiotic failures. Despite the species' regularity in meiosis and high pollen viability, there are still external factors to consider that impact its reproductive success, such as unsynchronized flowering periods between individuals of the same species (Deprá and Gaglianone 2018), fragmentation (Rosa et al. 2019), species distribution, and the lack of pollinators (Caires and Barcelos 2017; Meléndez et al. 2020). The landscape matrix where *G. americana* individuals are located in this study is highly fragmented, which restricts the movement of both pollen and pollinators.

An approach to reducing the effects of fragmentation is the creation of ecological corridors (De Araújo and Bastos 2019), agro-ecological landscaping, and the implementation of agroforestry and forestry systems (Santos et al. 2019; Marsden et al. 2020). These strategies can facilitate the movement of pollinators and seed dispersers between populations, thereby increasing the chances of reproduction and regulating gene flow. *G. americana* can be included in such strategies alongside other native fruit trees to attract animals that will directly work as pollinators and seed dispersers to contribute to reproductive success.

5. Conclusions

The pollen grains of *G. americana* are medium-sized, oblate-spheroidal, and 3-colporate with a reticulate exine. The colorimetric tests presented a significant difference and showed high pollen viability and meiotic regularity of the species. However, it is necessary to create means for conserving and restoring degraded areas, such as maintaining forest cover, creating ecological corridors, using native species in the landscape, and reducing pesticides and pollutants. Even with a large amount of viable pollen, there is no guarantee of success in the reproduction of the species due to various factors, mainly associated with fragmentation, genetic drift, reduced gene flow, and inbreeding.

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